THE NEUROPHARMACOLOGY OF A SLOWLY ADAPTING TYPE 1 SENSORY RECEPTOR

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ABSTRACT

The role of the Merkel cell-neurite complex in the transduction process in slowly adapting type 1 (SA1) cutaneous mechanoreceptors is unresolved. One hypothesis, based largely on the ultrastructure of Merkel cell-neurite complexes, suggests that chemosynaptic transmission occurs between the Merkel cell and its subjacent nerve terminal. This idea was investigated by mechanically stimulating SA1 mechanoreceptors exposed to pharmacologically active agents in several experimental preparations; an *in vivo* rat model, and *in vivo* feline isolated hind limb perfusion model and a novel isolated rat skin-nerve preparation.

After exposure to the calcium channel blockers Mg^{2+}, Cd^{2+} and verapamil hydrochloride there was a dose dependent decline in the response of the SA1 mechanoreceptors to mechanical stimulation. Given that an influx of Ca^{2+} ions is required for stimulus-secretion coupling, these results support the hypothesis of chemosynaptic transmission.

Immunohistochemical studies have shown that a met-enkephalin-like substance is associated with the dense cored vesicles in rodent Merkel cells. The idea that met-enkephalin was the transmitter substance in rat Merkel cell-neurite complexes was tested using the opiate antagonist naloxone and the agonist met-enkephalin in the isolated rat skin-nerve preparation. Met-enkephalin caused a dose dependent decline in the response of the SA1 mechanoreceptors to mechanical stimulation. This effect was antagonised by naloxone, indicating the presence of functional opioid receptors in the SA1 sensory receptor. However, this result indicates that met-enkephalin is not the excitatory transmitter substance in rat Merkel cell-neurite complexes, though it does have a modulatory role.

The results presented in this thesis support the hypothesis that chemosynaptic transmission is involved in the transduction process in the Merkel cell-neurite complex.

CHAPTER 1

INTRODUCTION.

INTRODUCTION

THE DISCOVERY OF SLOWLY ADAPTING TYPE 1 MECHANORECEPTORS

The first description of Merkel cells in the literature (Merkel,1875) and the correlation of slowly adapting afferent fibre activity with these components of slowly adapting type 1 (SA1) mechanoreceptors took almost a century (Iggo,1963a).This was despite the names given to the Merkel cell (Tastzellen) and the Merkel cell-neurite complex (Tastkorperchen) reflected Merkel's hypothesis that the Merkel cell-neurite complex converted physical stimuli applied to the skin to electrical activity in the adjacent nerve fibre. Further descriptions of Merkel cells were made by Ranvier (1880). In a review of peripheral nerve terminations,the very existence of Merkel cells was questioned (Weddell,Palmer and Pallie,1955) when they suggested Merkel cells were dendritic cells described by Billingham (1949). Improved histological techniques and the use of the electron microscope resolved this controversy. The first E.M. descriptions of Merkel cell-neurite complexes occurred in 1962 (Cauna,1962). They were then described in SA1 mechanoreceptors of cat hairy skin (Iggo and Muir,1963) glabrous skin of the opossum snout (Munger,1965) and sinus hair follicles (Andres,1966).

The slowly adapting afferent fibre response was described much earlier (Adrian and Zotterman 1926). Further descriptions of slowly adapting responses indicated that they were associated with highly sensitive

spot like areas of skin (Frankenhauser,1949; Maruhashi,Mizuguchi and Tasaki,1952; Hunt and McIntyre 1960). Iggo (1963b) divided slowly adapting mechanoreceptors into two types based on differences between their electrophysiological responses and the size of their receptive fields. Some had receptive fields of $>1mm^2$ (touch fields) and others receptive fields of $250um^2$ (touch spots). In another publication at this time, Iggo (1963a) correlated the responses of the "touch spots" with a distinctive structure in cat skin. This correlation of slowly adapting response with Merkel cells has been confirmed in subsequent reports (Tapper,1964; Iggo and Muir,1969).

Though it is now accepted that Merkel cells underlie the SA1 response,their physiological function is not certain. There are two hypotheses: 1) The Merkel cell is a primary receptor cell, responding to mechanical deformation by the secretion of a transmitter substance (Merkel,1875; Andres,1966; Iggo and Muir,1969; Horch, Whitehorn and Burgess,1974; Hartschuh and Weihe 1980). 2) The Merkel cell is an abutment, making the mechanosensitive ending more sensitive to mechanical deformation (Smith,1977; Gottschaldt and Vahle-Hinz 1981, 1982). In the remainder of the introduction the literature will be reviewed to see whether the evidence supports either of the above hypotheses.

MORPHOLOGY

Merkel cell-neurite complexes comprise a distinctive sensory structure in the epidermis of hairy skin called SA1 mechanoreceptors or touch domes (Fig1.1). Each SA1 mechanoreceptor is a hemispherical dome of 150-250um diameter containing a fine capillary network (Iggo 1963a). There are 25-150 Merkel cell-neurite complexes within a single touch dome (Nurse, Mearow, Visheau, Holmes and Diamond,1983) which lie beneath a layer of epidermis approximately four epithelial cells thick (Iggo and Muir 1969). Each Merkel cell has a diameter of 9-16um and contains a large polylobulated nucleus. The Merkel cell-neurite complexes are orientated such that their nuclei are parallel to the skin surface and the nerve plate lies on the dermal side.

Each touch dome is innervated by a single myelinated afferent fibre of 7-16um diameter (Iggo,1963a; Frankenhauser 1949). There is extensive branching of this parent axon in the dermis and one axon may innervate several touch domes (Hunt and McIntyre 1960a; Iggo 1963a). The afferent fibre loses its myelin within 10um of the Merkel cells and terminates as a flattened disc 10um long and 1um thick closely apposed to the dermal side of the Merkel cell.

Electron microscopical examination shows that the nerve plates contain numerous mitochondria and a variable degree of glycogen particles of different sizes (Hartschuh

FIGURE 1.1

This diagram shows the structure of an SA1 mechanoreceptor present in cat hairy skin as seen in light microscope sections (from Iggo and Muir, 1969).

FIGURE 1.2

This diagram shows the structure of a Merkel cell-neurite complex as seen at the electron microscope level (from Iggo and Muir, 1969).

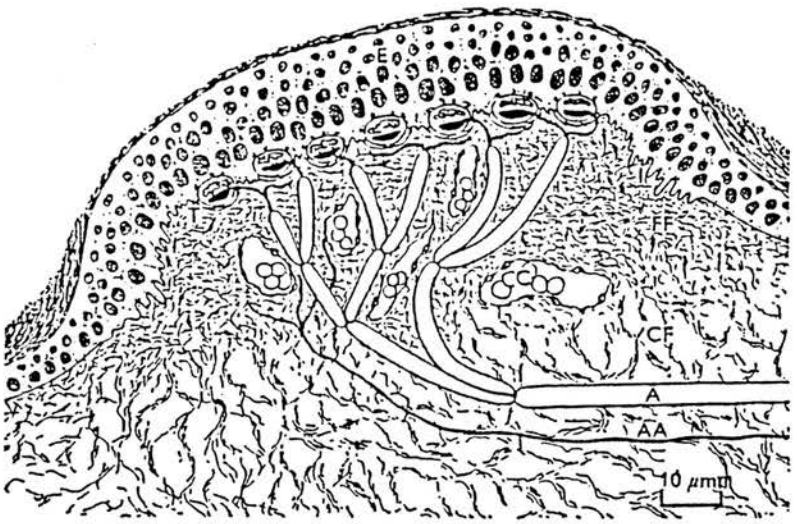


fig. 1. A diagram showing the structure of a touch corpuscle as seen in light microscope sections. A, single myelinated axon; AA, non-myelinated axons; E, thickened epidermis of the touch corpuscle; FF and CF, fine and coarse bundles of collagen fibres; I, extensive indentations of the dermis by epidermis at the periphery of the corpuscle: T, tactile cell and its associated nerve plate; C, capillary.

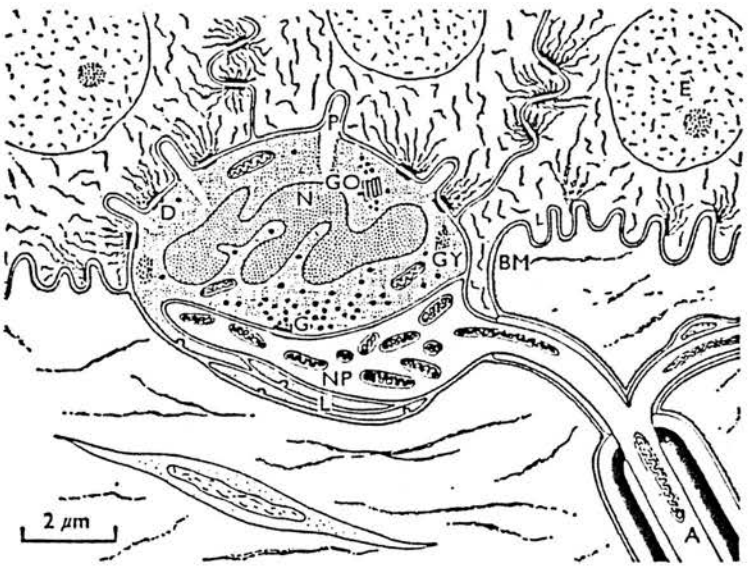


fig. 2. A diagram showing the structure of a tactile cell and its associated nerve plate. A, myelinated axon; BM, basement membrane; D, desmosome; E, epithelial cell nucleus; G, granular vesicles in the tactile cell near a junction with the nerve plate, NP; GO, Golgi apparatus; GY, glycogen; L, lamellae underlying the nerve plate; N, multilobulated nucleus; P, cytoplasmic process from the tactile cell.

and Weihe 1980). An ultrastructural representation of a Merkel cell-neurite complex is shown in Fig1.2. The Merkel cells are connected to neighbouring keratinocytes by desmosomes. The membrane is smooth but on its superficial side there are several microvillous processes 1.5um long and 0.3um diameter with fine filamentous cores which penetrate between the overlying keratinocytes (Iggo and Muir 1969). These may be analagous to the stereocilia of vertebrate hair cells ; the stereocilia are tubular projections of the cell membrane arising from the cell surface and have actin filaments running down their length (Flock and Cheung 1977). The composition of the filaments in Merkel cells is unknown. There are thickened regions of Merkel cell and nerve plate membrane which have granular material between them (Iggo and Muir 1969; Chen, Gerson and Meyer 1973), characteristics normally associated with chemical synapses. The cytoplasm of the Merkel cell contains ribosomes, a sparse rough endoplasmic reticulum, Golgi apparatus, lysosomes and glycogen particles at the poles of the nucleus. The most distinctive structures in Merkel cells are the membrane bound, electron dense granules (diameter 80-120um). Generally the granules are concentrated in those parts of the cell facing the basal lamina and the nerve terminal, though some may be found in the superficial half of the cell (Iggo and Muir 1969). Sometimes the granules are found closely apposed to the specialised regions between the Merkel cell and the nerve endings described above (Andres,1966; Iggo and Muir 1969) where they have also

been seen to fuse (Chen, Gerson and Meyer 1973). These observations have been confirmed in later work (Andres and von Duering 1973; English 1974; Smith 1977; Mihara, Hashimoto, Ueda and Kumakiri 1979; Hartschuh and Weihe 1980). Conversely other authors have found no evidence suggesting a synaptic relationship between the Merkel cell-neurite complex (Munger,1965; Smith 1970; Hashimoto 1972b; Winkleman 1977). Different post fixatives and stains critically affect whether synaptic features are visible or not (Hartschuh and Weihe 1980). Using new methodology, Smith (1977) revised his findings and stated that specialised junctions did exist and granules could be seen fusing there. Hartschuh and Weihe (1980) suggested the granules function as synaptic vesicles. So far no clear synaptic vesicles, which are generally considered a feature of synapses, have been observed. The functional significance of clear vesicles has been questioned (Gray 1977; Tauc 1982).

Hence, Merkel cell-neurite complexes show ultrastructural features characteristic of cells having a neurosecretory function.

MORPHOGENESIS

Currently there are two hypotheses concerning the origin of Merkel cells. One suggests that Merkel cells are neural crest migrants and the other that Merkel cells develop from epithelial cells in the epidermis.

The first is supported by observations of Merkel cells in the dermis of human foetuses (Breathnach 1971

Hashimoto 1972a). These authors have assumed that the Merkel cells are migrating from the dermis into the epidermis but this assumption has not been confirmed. Comparisons between Merkel cells and cells classified under the APUD (amine, precursor-uptake, decarboxylase) system, some of which are derived from the neural crest, revealed morphological and biochemical similarities (Winkleman 1977). Ontogenetic studies of developing Grandry and Merkel corpuscles in birds have shown that these cells are derived from the neural crest (Saxod 1978b; Ide and Munger 1978). However it is unlikely that mammalian Merkel cells and Grandry and Merkel corpuscles in birds are identical since the latter are rapidly adapting. In amphibian larvae removal of neural crest tissue did not affect the development of Merkel cells (Tweedle 1978).

Support for the second hypothesis comes from observations of cells in the epidermis transitional in appearance between Merkel cells and keratinocytes (Smith 1970; English 1974, 1977b). Other studies investigating ontogenetic development of mammalian Merkel cells also suggest an epidermal origin (Lyne and Hollis 1971; Pac, 1984). Recently, indirect immunofluorescence techniques revealed that Merkel cells contain keratin intermediate filaments and desmosomal protein typical of epithelial cells (Saurat and Didierjean 1984; Saurat, Didierjean, Skalli, Siegenthaller and Gabbiani 1984) but distinct from keratinocytes (Moll, Moll and Franke 1984; Ortonne and

Darmon 1985; Moll, Moll and Franke 1986).

Though it was hoped that investigations into the developmental origin of Merkel cells would give some indications about their physiological function this has not been the case.

MERKEL CELL DEGENERATION AND REGENERATION

Merkel cell-neurite complexes show degenerative changes such as a decrease in the number of Merkel cells and granules and a thinning of the epithelial layers over the dome crown after nerve section or nerve crush (Brown and Iggo 1963; Burgess, English, Horch and Stensaas 1974; English 1977a; Nurse, Macintyre and Diamond 1984). At various stages of regeneration distal to the crush, mechanical stimulation elicited non-specific responses. Only when regenerated fibres reformed Merkel cell-neurite complexes did the typical slowly adapting responses return (Brown and Iggo 1963). Studies on newborn kittens support these findings; only when numerous Merkel cells appeared in the epidermis was a sustained response to mechanical stimulation possible (Kasprzak, Tapper and Craig 1970). Similar morphological and physiological effects occur after denervation in other sensory systems. A generalised degeneration of the gustatory epithelium was the result of transection of the glossopharyngeal nerve in the rat (Guth 1971). Nerve crush caused loss of osmosensory ability in the cat carotid body. Ultrastructural examination revealed that the appearance of nerve endings adjacent to the glomus-sustentacular cell complex coincided in time with

re-establishment of osmosensory ability (Eyzaguirre and Fidone 1980).

Treatment with the vinca alkaloids colchicine and vinblastine caused a decreased number of, and degeneration in, Merkel cells, an effect similar to nerve transection (Chelyshev and Vinter 1983). The authors suggested that axonal blockade prevented the secretion of trophic chemical factors needed to maintain Merkel cells. After the application of vincristine to rat touch domes there was an increase in the threshold to mechanical stimulation without any increase in response latency suggesting the drug is capable of affecting the function of the mechanoreceptor before there is interference with impulse propagation (Leon and McComas 1984). Some investigators have reported no changes in Merkel cells after denervation (Smith 1967; Hartschuh and Weihe 1977). This discrepancy may be explained by sampling difficulties associated with E.M. studies. Denervation did not affect amphibian Merkel cells (Cooper, Diamond and Turner 1977) though the granule content was reduced in another study (Tweedle 1978).

Both crushed and transected type 1 fibres preferentially regenerated to old receptor loci (Burgess, English, Horch and Stensaas 1974). This topographical specificity appears to reflect intrinsic properties of the site rather than guidance of regenerating axons along Schwann cells in the distal stump (Horch 1982). It has been suggested that a chemical associated with the basal membrane is responsible in regenerating muscle fibres and

nerves(Guthrie, 1986).

DISCHARGE PATTERN

SA1 mechanoreceptors have a characteristic irregular firing pattern and are normally silent in the absence of a mechanical stimulus (Tapper 1965; Iggo and Muir 1969). They have low mechanical thresholds (1-5um displacement) though afferent activity does not persist at these displacements. The response can be divided into two major components (Tapper 1965; Iggo and Muir 1969): 1) The dynamic component which has a relatively high frequency and occurs during indentation of the skin. The discharge frequency is determined by both the velocity and the amplitude of the displacement (Iggo and Muir 1969). 2) The static component of the response occurs during a maintained mechanical displacement. The response frequency can be related to the stimulus amplitude by the power function $R=kS^b$ where R is the response frequency, k is a constant and S is the stimulus amplitude raised to the power of b (Werner and Mountcastle 1965; Tapper 1965). This phase of the response can be further subdivided into two components. Initially there is a rapidly adapting phase, followed by a more slowly adapting phase (Tapper 1965; Iggo and Muir 1969). During the static component the inter spike intervals (ISIs) are variable especially after the initial rapidly adapting phase. Iggo and Muir (1969) established that, apart from intervals shorter than 45 ms, the ISIs had an exponential distribution, implying that

ISIs longer than 45 ms occur randomly and independently. This pattern of firing was attributed to the presence of multiple impulse generating sites within the receptors. Indeed this is consistent with the concept of separate generators since each Merkel cell is innervated by a single expanded nerve ending of a branch of the main axon.

Assuming that SA1 mechanoreceptors did contain multiple impulse generating sites, Horch, Whitehorn and Burgess (1974) suggested two models to explain this characteristic discharge pattern based on the morphological characteristics of the receptor. The first model assumed that each Merkel cell-neurite complex acted as an independent oscillator, each of which generated a regular firing pattern. The irregular discharge pattern observed in the afferent fibre was due to mixing of the regular firing patterns. This model assumed that the impulses produced at one site did not influence those produced at another since each oscillator was completely independent. The second model assumed that each impulse generating site had an irregular firing pattern and that resetting of one site by another was possible. They found that the second model best described the firing pattern of SA1 mechanoreceptors and concluded that the most likely cause of such a pattern was the release of a transmitter substance from the Merkel cells. They suggested that the relationship between the Merkel cell and the adjacent nerve terminal was functionally related to the characteristic firing pattern of SA1 mechanoreceptors. Therefore, analysis of the discharge pattern of SA1

mechanoreceptors could support the concept of transmitter release from Merkel cells.

In feline sinus hair follicles, Merkel cell-neurite complexes followed vibration frequencies up to 1500Hz in a 1:1 relationship and the receptor delay was 0.3 ms (Gottschaldt and Vahle-Hinz, 1981). These authors argued that each of these parameters was too fast for chemosynaptic transmission. However, at the mammalian neuromuscular junction the interval between the maximum rise of intracellular spike potential and the beginning of transmitter release was in the region of 0.2 ms (Hubbard and Schmidt 1963). The time between Ca^{2+} entry and the start of the synaptic potential in a stimulated squid giant axon could be as short as 0.2ms (Llinas, 1977). It is established that chemical synapses exist in both of these preparations. Hence the argument that 0.3 ms is too short for chemosynaptic transmission becomes redundant. Analysis of the discharge pattern of SA1 mechanoreceptors supports the concept of transmitter release from Merkel cells.

THE SEARCH FOR POTENTIAL TRANSMITTERS

Since the suggestion that SA1 mechanoreceptors respond to mechanical stimulation by the release of a transmitter substance (Merkel 1875; Andres 1966; Iggo and Muir 1969; Hartschuh and Weihe 1980) much effort has been put into identifying possible transmitters. Most of the work has involved application of immunohistochemical

techniques to discover the contents of the granules. Another approach has been the application of drugs indirectly to the SA1 mechanoreceptors.

Since the granules resemble the monoamine storing organelles of chromaffin cells it was assumed that they might also contain biogenic amines such as noradrenaline or 5-hydroxytryptamine (5-HT). Neither fluorescence techniques nor the Falk method for staining catecholamines have given positive results (Smith, 1967; Hartschuh and Grube, 1979). Repeated reserpine administration had no effect on either the response or the ultrastructure of the touch domes (Smith, 1967; Iggo and Muir 1969). Administration of the amine precursors L-DOPA AND L-5-HT and the monoamine oxidase inhibitor harmaline had no effect on monoamine metabolism (Hartschuh and Grube, 1979). Since all histochemical and pharmacological attempts to determine monoamine metabolism in Merkel cells failed, it is unlikely that those currently identified are involved in the transduction process.

Smith and Creech (1967) found no drugs which could elicit spontaneous activity in the afferent fibres of SA1 mechanoreceptors. The only drug which increased the number of action potentials in response to a standard mechanical stimulus was nicotine which briefly stimulated before blocking the receptor; lobeline produced only a block. Local anaesthetics also blocked the response presumably acting on the afferent fibre. Solutions of 10^{-2}M K^{+}, Ca^{2+} and Mg^{2+} chloride also blocked the response. Extracts from

cat sinus hair follicles were injected into other similar receptors but had no effect on the responses of SA1 mechanoreceptors (Smith, 1977).

Winkleman (1977) suggested that the Merkel cells might produce a polypeptide functioning as a transmitter. Subsequently a met-enkephalin-like immunoreactivity was demonstrated at the light microscope level in rodent Merkel cells (Hartschuh, Weihe, Buchler, Helmstaeder, Feurle and Forssmann, 1979). Since the strongest immunoreaction occurred in the areas of highest granule density, they suggested that the granules were the site of the immunoreaction. They suggested that the Merkel cell may be a member of the paraneuronal system and thus a neurosecretory cell. This hypothesis was tested by observing the effect of intra-venously administered naloxone, an opiate antagonist on the response of feline sinus hair follicles to mechanical stimulation (Gottschaldt and Vahle-Hinz, 1982). Even at doses where the drug would act non specifically, there was no effect. The authors concluded that Merkel cells did not function as neuroreceptor cells. However, it was subsequently reported that the met-enkephalin-like immunoreaction was peculiar to rodent Merkel cells (Hartschuh, Weihe, Yanaihara and Reinecke, 1983). This result makes the experiment of Gottschaldt and Vahle-Hinz inappropriate since more recent studies suggest that Merkel cells found in upper lip regions of guinea pigs contain opioids derived from proenkephalin and / or prodynorphin rather than met-enkephalin per se (Hartschuh and Weihe, 1988).

Merkel cells in cat, dog, pig and man were immunoreactive to vaso-active intestinal polypeptide (VIP) and again the strongest immunoreaction was in the area of highest granule density (Hartschuh, Reinecke, Weihe and Yanaihara, 1984). Immunoreaction to PHI and PHM, precursors of VIP, has been demonstrated in pig snout skin (Hartschuh, Weihe, Yanaihara and Yanaihara, 1986; Hartschuh and Weihe, 1988). The unique presence of opioid peptides in rodents possibly indicates a separate evolutionary line. Calcitonin-gene related peptide (CGRP) and substance P (SP) immunoreactivity have also been demonstrated in the snout skin of the pig (Hartschuh and Weihe, 1988). They have a wide distribution the nervous system and co-exist with each other in primary sensory neurones (Lundberg and Hokfelt 1986) and with prodynorphin derived opioids in guinea pigs (Weihe, Hartschuh and Weber, 1985). At present the functional significance of this complex array of peptides in Merkel cells is not certain. Both met-enkephalin and VIP are transmitter candidates (Kosterlitz and McKnight, 1981; Fahrenkrug and Emson 1982) supporting the concept that Merkel cells are neuroendocrine cells of the diffuse neuroendocrine system (DNES) (Pearse, 1986). Merkel cells exhibit other characteristics of cells classified under the DNES. They contain neurone specific enolase (NSE) (Gu, Polak, Tapia, Marangos and Pearse, 1981), synaptophysin and chromogranin A (CGA) is associated with the granules (Hartschuh and Weihe, 1988). It has been suggested that NSE facilitates interaction with positively charged cytoskeletal proteins (Forss-

Petter, Danielson and Sutcliff 1986). Synaptophysin may be involved in the process of vesicle formation and exocytosis (Wiedenmann, Franke, Kuhn, Moll and Gould 1985). CGA may be involved in packaging of regulatory peptides into secretory granules (Rosa, Hille, Lee, Zanini, Camilli and Huttner, 1985) or in the sequestration and mobilization of Ca^{2+} from secretory vesicles during stimulus-secretion coupling (Benedum, Baeuerle, Konecki, Frank, Powell, Mallet and Huttner, 1986). These results suggest that Merkel cells may be neurosecretory cells.

The role played by the granules in the transduction process has been investigated using another technique. If granules do contain a transmitter substance, then its synthesis, storage and release will be O_2 sensitive processes. Under conditions of extreme hypoxia, SA1 mechanoreceptors failed to respond to mechanical stimulation and this was correlated with almost total loss of granules from the Merkel cells in cats (Anand, Iggo and Paintal 1979). Further experiments showed the effects of hypoxia could be reversed within 30sec by replacing the N_2 around the limb with O_2 and reapplication of N_2 caused the response to fail again (Findlater, Iggo, Anand and Paintal, 1987). A quantitative study of these effects was made using an isolated hind limb model in the cat in which SA1 mechanoreceptors could be made reversibly hypoxic (Cooksey, Findlater and Iggo, 1983 ; Iggo and Findlater, 1984; Findlater, Cooksey, Anand, Paintal and Iggo 1987). Similar results were observed. In addition they showed

that though SA1 mechanoreceptors failed to respond to mechanical stimulation, the afferent fibre still responded to electrical stimulation; thresholds for stimulation and conduction velocities were unaltered between normoxic and hypoxic conditions. These results suggest that granules are required for the normal function of SA1 mechanoreceptors and that the transduction process is O_2 dependent.

Calcium is required for the release of various substances, including neurotransmitters, from secretory cells. This has been demonstrated for the release of acetylcholine at the frog neuromuscular junction (Katz and Miledi, 1965, 1967a, 1967b) and the release of catecholamines from the adrenal medulla (Baker and Knight, 1981). Calcium binding sites have been identified in the membrane of the dense cored vesicles within the glomus cells of the cat carotid body (Hess, 1977; Hansen and Smith, 1979). There are structural similarities between Merkel cell-neurite complexes and hair cells of the vertebrate auditory system. The cytoplasmic processes on the apical surface of the Merkel cell may be analagous to the stereocilia , which contain actin filaments, on the apical surface of hair cells. Displacement of these stereocilia is the first step in the transduction process in hair cells and it is considered likely that mechanical energy is transduced to an electrical signal through mechanically gated ionic channels named mechano-electric transducer channels (m-e.t channel) (Ohmori, 1987). The

site of the m-e.t channel is not established. Hudspeth (1982) suggested that it exists at the tip of each stereocilium. Angular displacement of the hair bundle about its point of insertion into the cuticle rather than absolute displacement of the hair bundle was found to be crucial for m-e.t (Ohmori, 1987) which suggests the m-e.t channel lies at the base of the stereocilium. Calcium binding sites are associated with the stereocilia of vertebrate hair cells (Moran, Carter and Asher, 1981). Ohmori (1985) demonstrated that extracellular Ca^{2+} was required for activation of the m-e.t channel in chick vestibular hair cells. The conductance of these channels was reduced by Co^{2+} and La^{2+} (Ohmori, unpublished). D600 also blocks hair cell transduction (Jorgensen, 1979). Mechanical displacement of the cytoplasmic processes in Merkel cells may be the initiating step for transduction in SA1 mechanoreceptors. This was first suggested by Iggo and Findlater in 1984. Using the feline isolated hind limb perfusion model, they produced preliminary results which suggested Co^{2+} and verapamil blocked the response of SA1 mechanoreceptors to mechanical stimulation while the response of the afferent fibre to electrical stimulation was not affected (Cooksey, Findlater and Iggo, 1984).

From the review of the literature it is apparent that the role of Merkel cell-neurite complexes in the transduction mechanism of SA1 mechanoreceptors is still unresolved. I decided to use a direct pharmacological approach in an attempt to help answer this question. Since Merkel cell-neurite complexes are not readily accessible,

due to their location in the epidermis, they do not easily lend themselves to pharmacological manipulation and up till now the indirect applications of drugs described above have been employed. Major drawbacks of these methods are the uncertainty of the concentration of drug reaching the intended site of action and the potential problem of the drug acting on other systems while en route. To overcome these problems I developed an isolated rat skin-nerve preparation which could be superfused with solutions containing known concentrations of drugs. Further advantages were requiring smaller amounts of drugs and the avoidance of skin creep and recovery problems (Pubols, 1982b).

Using this preparation, the effect of magnesium, cadmium and verapamil, drugs which interfere with calcium entry, were measured. The hypothesis that met-enkephalin is the transmitter substance in rodent Merkel cells was also tested using the opioid drugs, naloxone and met-enkephalin. These experiments were complemented by in vivo experiments where appropriate.

CHAPTER 2

METHODS.

METHODS

2.1 GENERAL PREPARATION FOR IN VIVO RAT EXPERIMENTS

Anaesthesia was induced in Albino-Wistar rats (300-420g) using a 4% mixture of halothane in oxygen. Following cannulation of the external jugular vein, anaesthesia was maintained using 0.35ml/kg of a 1% chloralose, 20% urethane (w/v) mixture in 0.9% saline. Supplementary doses were given as required.

The trachea was cannulated to maintain a patent airway. Arterial blood pressure recordings were made via the carotid artery. The minimum mean diastolic blood pressure accepted was 80mmHg. The body temperature was kept constant at 38^{o}C by placing the animal on a thermostatically controlled electric blanket. Arterial blood samples (0.5ml) were taken for measurement of pO_2, pCO_2 and pH.

Hair was removed from the gluteal region of the trunk by shaving and then using a depilatory agent (Immac). The left hind limb was fixed in a secure position by anchoring the foot to a wooden block using a plaster of Paris bandage. The head was held in position by a head holder and a piece of polystyrene placed under the axillae thus raising the thorax and avoiding transmission of general movement caused by respiration to the recording site. A dorsal incision (~40mm long) 10mm to the right and parallel to the midline was made in the lumbar region. The skin flaps were reflected to expose the 4th and 5th dorsal

lumbar cutaneous nerves. The superficial fascia was removed and the nerve dissected free from the surrounding tissue. Threads were passed through the reflected skin flaps and tied around a metal ring placed over the incision site, thus forming a pool. An earth electrode was inserted into the paraspinal muscles and tied in place.

Nerve dissection, signal recording, identification of SA1 mechanoreceptors and mechanical stimulation are described below.

The possible involvement of the met-enkephalin-like immunoreactivity associated with the granules within Merkel cells in the transduction process was investigated using the opiate antagonist naloxone. SA1 mechanoreceptors were stimulated once per minute for 10sec for a period of 30 minutes. After the 5th stimulus, naloxone (0.1, 0.5 and 1.0mg/kg in 0.5ml saline) was administered as a bolus via the external jugular vein. The effect of naloxone was assessed by comparing successive responses to stimulation in animals which received no drug with those receiving one of the doses specified above. Control animals were also compared with those receiving saline alone. The reason for giving naloxone or saline after the 5th stimulus was to ensure that the receptor under investigation was typical.

2.2 NERVE DISSECTION

Once the nerve had been dissected free from the surrounding tissue, a black perspex plate (5mm wide) was placed beneath it. Paraffin B.P. warmed to 37^{o}C was poured

into the pool. Subsequent dissection was carried out with the aid of a binocular dissecting microscope which allowed magnification between x6 and x40 (Carl Zeiss).

The epineurium around the nerve was removed for ~10mm using fragments of razor blades held in pintongs. Using fine needles, strands of nerve fibres were dissected out from the main bundle. A strand of fibres was placed over one of a pair of Ag/AgCl hook electrodes using fine forceps. A narrow strand of connective tissue was placed over the other hook electrode to act as a reference. Any tissue fluid oozing from the tissues surrounding the nerve was removed with a Pasteur pipette because it would short out electrical signals.

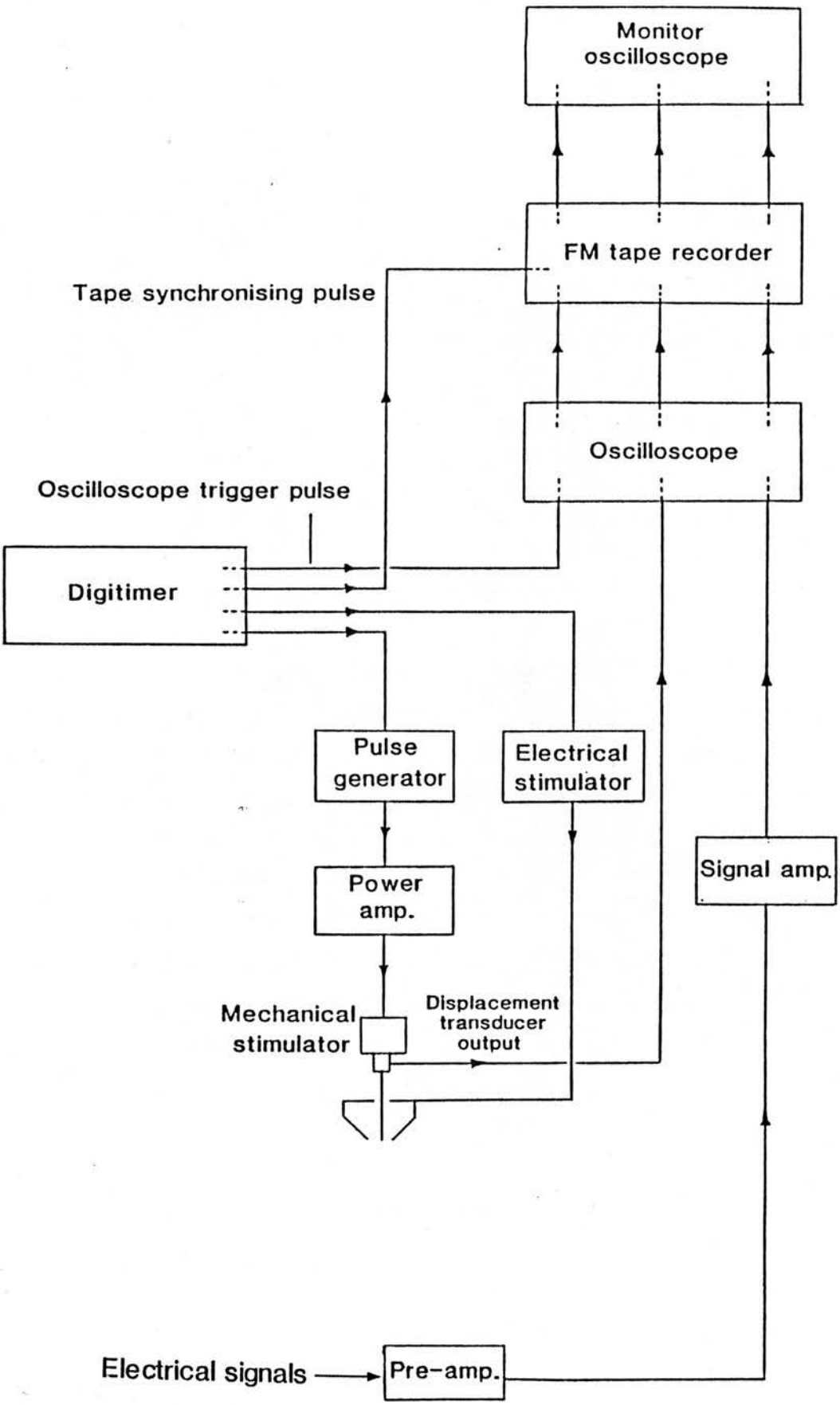
2.3 SIGNAL RECORDING AND IDENTIFICATION OF SA1 MECHANORECEPTORS

Signals from the electrodes were initially passed through a differential pre-amplifying head stage (input impedance >10 M ohms, gain x10) and then fed into a differential amplifier. The amplifier filters were set to give a bandwidth of 0.1-3.0 KHz and a gain of x1000 was used. Amplified signals were fed into a loudspeaker, displayed on an oscilloscope (Tektronix 5103N) and led into one of eight channels of an FM tape recorder (Ampex PR-500). Figure 2.1 shows the recording arrangement.

The receptive fields of the afferent fibres were identified by moving glass or perspex probes over the skin surface. The SA1 mechanoreceptors could be recognized over the loudspeaker by 1) their characteristic high

FIGURE 2.1

This diagram illustrates the general methods used for mechanical stimulation and the recording of electrical signals in response to this stimulation. The methods used for electrical stimulation in the cat experiments is also shown.


Monitor oscilloscope
FM tape recorder
Tape synchronising pulse
Oscilloscope
Oscilloscope trigger pulse
Digitimer
Pulse generator
Electrical stimulator
Signal amp.
Power amp.
Mechanical stimulator
Displacement transducer output
Electrical signals
Pre-amp.

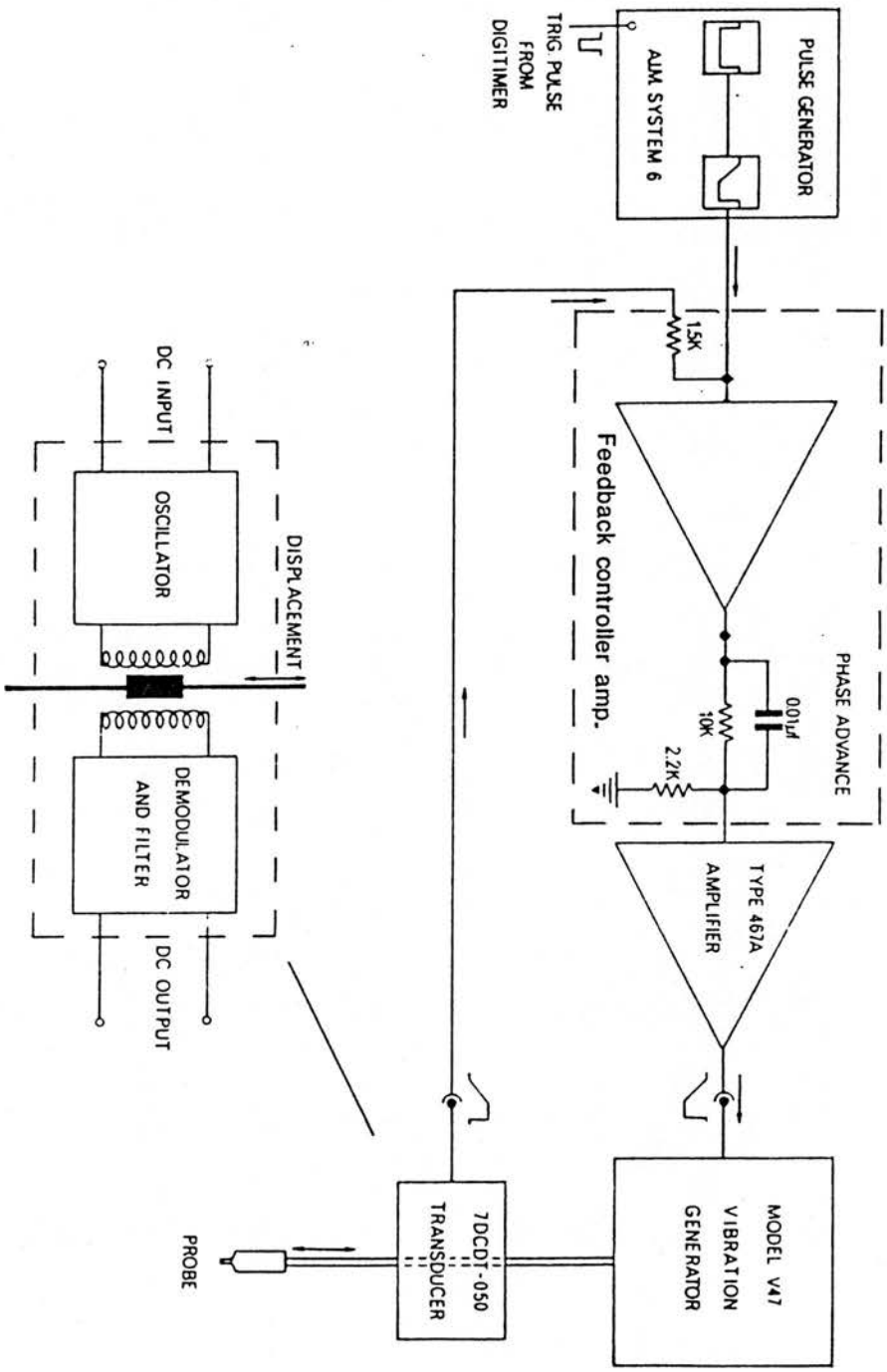
frequency discharge as the probe passed over them, 2) their sustained response to continuous stimulation and 3) their discrete and extremely small receptive fields. They were then located with the aid of the dissecting microscope and their presence confirmed by the use of the mechanical stimulator.

2.4 MECHANICAL STIMULATION

Figure 2.1 shows the general arrangement. A master oscillator (Digitimer, Devices Ltd.) triggered the oscilloscope and 100ms later triggered the pulse generator (AIM PWD 103A). The pulses produced by this unit could be varied in duration between 25ns and 10s. Pulses of the desired duration were fed to a module (AIM VRF 107) capable of varying their rise and fall time independently at any voltage level between -20V and +20V. Pulses were then passed to a mechanical stimulator system, consisting of a feedback controller amplifier, a mechanical stimulator and a probe displacement transducer, (Fig. 2.2). The output of the feedback controller amplifier was fed into a power amplifier (Hewlett Packard, 467A) which produced a current capable of driving the mechanical stimulator (model V47 Goodmans Industries Ltd.). The stimulator worked on the same principle as a moving coil loudspeaker and followed the shape of the input waveform, turning electrical potential into mechanical displacement. A thin steel rod was connected to the moving core of the stimulator and its movement was monitored by a displacement transducer (7DCT, Hewlett Packard). This gave

FIGURE 2.2

This diagram shows the arrangement of the mechanical stimulator system. A trigger pulse from the Digitimer entered the pulse generator. The output of the pulse generator was amplified and passed onto the mechanical stimulator. The probe displacement produced by the mechanical stimulator was monitored by a probe displacement transducer, the output of which was fed back into a controller amplifier. This meant that any deviations could be corrected and this ensured that the mechanical stimulator followed the output of the pulse generator faithfully.



MECHANICAL STIMULATOR SYSTEM

7DCDT TRANSDUCER SCHEMATIC DIAGRAM

a DC output proportional to the linear displacement of the probe. This output was fed back into the feedback controller amplifier to improve the tracking of the electrical signal by the mechanical stimulator.

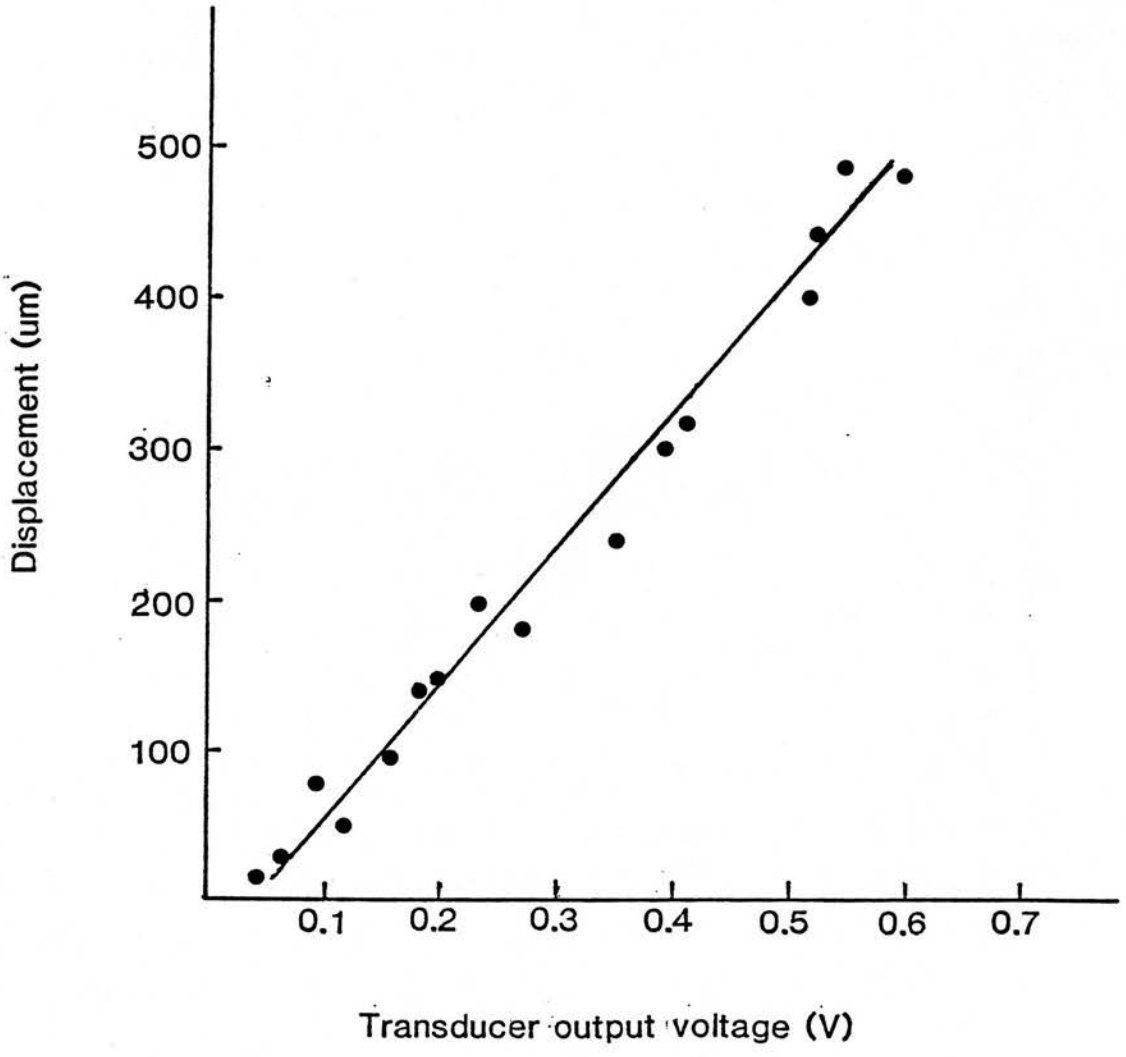
The probe displacement was measured with a calibrated eye-piece graticule fitted into the dissecting microscope. These measurements were taken at different transducer output voltages which were displayed on the oscilloscope. Corresponding values of probe displacement (microns) and output voltage (mV) were used to draw a calibration curve (see Fig. 2.3) which was used to determine displacement values in all experiments. The rate of displacement of the probe was obtained from the oscilloscope by measuring the time taken for the probe to reach its final displacement. These values are given under general procedures for the various experimental types.

The point of a 23G needle was removed and the needle plugged with solder. This was rubbed down with glass paper to produce a smooth tip of diameter 500 microns and attached to the steel rod in the stimulator . Using the dissecting microscope the probe was placed on an SA1 mechanoreceptor, normal to its surface, until a response was just obtained. This ensured that the receptor was displaced by the desired amount.

The oscilloscope trigger , tape synchronising pulse and displacement transducer output were all recorded on the FM tape recorder.

FIGURE 2.3

This diagram represents the calibration curve for the mechanical stimulator. It was used to calculate the probe displacement (um) from the transducer output voltage (v) displayed on the oscilloscope.


Displacement (um)
500
400
300
200
100
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Transducer output voltage (V)

Stimulus duration, interstimulus intervals and stimulus amplitudes were chosen with reference to two criteria: 1) they gave as high a degree of stationarity as possible (i.e. they did not obviously fatigue the receptor); 2) they suited the time course of action of the drug given its route of administration (i.e. took into consideration the time taken for the drug to exert its effects and its rate of metabolism).

2.5 GENERAL PREPARATION FOR IN VIVO CAT EXPERIMENTS

Cats (1.8-2.4kg) were initially anaesthetized with a 4% mixture of halothane in oxygen. Following cannulation of the left femoral vein, anaesthesia was maintained using 70mg/kg of a 10mg/ml solution of a-chloralose in 0.9% saline. Supplementary doses were given as required.

The animal was placed on its dorsal surface on a thermostatically controlled electric blanket. The trachea was cannulated to maintain a patent airway.

The skin overlying the saphenous nerve to the foot of the right hind limb was shaved. An incision was made through the skin over the saphenous nerve from the lower edge of the abdominal wall to a point just proximal to the knee. A pad of fatty tissue overlying the femoral blood vessels as they passed from beneath the abdominal wall was carefully removed. The femoral artery and vein were dissected free from the surrounding tissue for a length of 15-20mm as close to the abdominal wall as possible. All large branches of these vessels were located and tied off.

Heparinized cannulae were placed in the central and peripheral ends of each of these vessels and interconnected by a system of taps and tubing (see Fig.2.4).

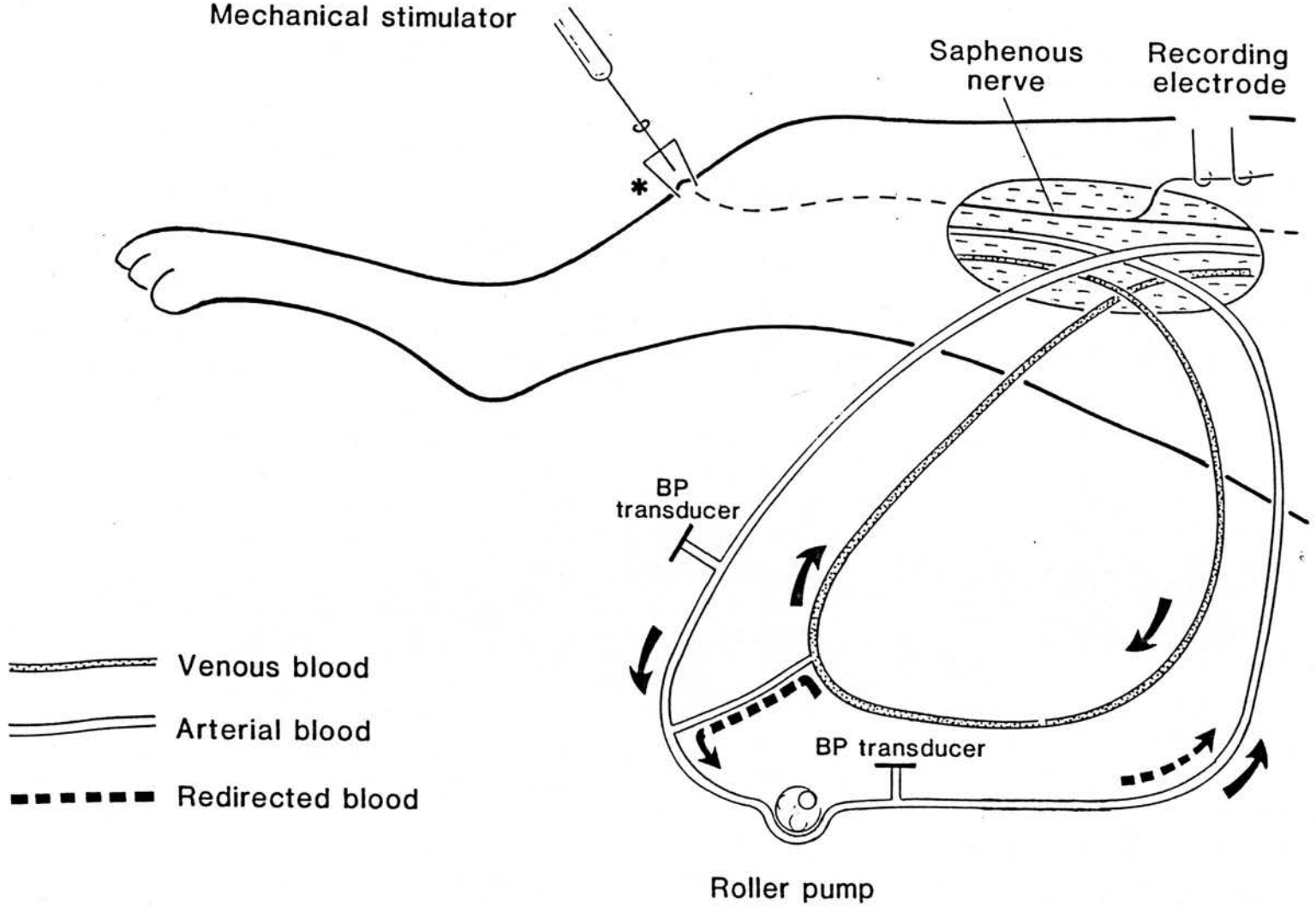
The arterial blood pressure (a.b.p.) of the animal was monitored by a transducer via the cannula placed in the central end of the femoral artery. This arterial blood was passed back down the limb by a roller pump (MHRE Mk3 Flow Inducer, Watson Marlowe Ltd.). The pressure of the blood at the pump output was also measured by a blood pressure transducer. By adjusting the speed of the pump it was possible to return the blood to the limb at the same mean pressure as arterial blood pressure. Venous blood returned passively from the limb to the general circulation. To isolate the limb circulation from the general circulation, the central end of the femoral artery could be occluded and the venous return redirected into the peripheral end of the artery.

By isolating the limb circulation, it was possible to introduce drugs of interest which may modify the response of SA1 mechanoreceptors without having the complications that introduction of such drugs into the general circulation may have brought. Though this procedure made the limb hypoxic, it was already known that this had no effect on the responses of SA1 to mechanical stimulation mechanoreceptors provided the atmosphere around the limb contained oxygen (Iggo and Findlater, 1984; Findlater et al, 1987).

FIGURE 2.4

This diagram illustrates the isolated hind limb perfusion model in the cat. Arterial blood pressure was monitored via the cannula in the central end of the femoral artery. This arterial blood was passed back down the limb via a roller pump. The blood pressure was monitored at the pump output and by adjusting the speed it was possible to return blood to the limb at the same mean pressure as arterial blood pressure. Venous blood returned passively from the limb to the general circulation. To isolate the limb circulation from the general circulation, the central end of the femoral artery was occluded and the venous return redirected into the peripheral end of the femoral artery. Drugs could then be introduced into the isolated limb circulation.

The method used to stimulate the SA1 afferent fibres electrically is also shown. Two electrodes were placed in the skin on either side of the touch dome and connected to an electrical stimulator. Electrical stimulation preceded mechanical stimulation.


Mechanical stimulator
Saphenous nerve
Recording electrode
*
BP transducer
BP transducer
Venous blood
Arterial blood
Redirected blood
Roller pump

The limb was then fixed to a wooden block using a plaster of Paris bandage. Threads were passed through the skin flaps of the incision and a paraffin pool constructed. Nerve dissection, signal recording and mechanical stimulation were as described above (Sections 2, 3 and 4). Once a suitable SA1 mechanoreceptor had been located, the circulation to the limb was isolated. The receptors were mechanically stimulated twice per minute for 5s for a total time of ten minutes (i.e. 20 stimulations).

Drugs used in this preparation were verapamil hydrochloride (100) and cadmium chloride (0.5, 1.0 and 2.5mM). Drug concentrations were calculated on the assumption that the blood volume in the limb was 20ml and they were given in 1.0ml of saline.

In these experiments the ability of the afferent fibre to conduct impulses after receptor failure was tested. Two silver chloride electrodes were implanted in the skin on either side of the touch dome. These were connected to the output terminals of a constant voltage electrical stimulator (Devices, 2533). The voltage required to elicit an action potential in the afferent fibre in 50% of the test stimulation applied was determined. All subsequent electrical stimulations applied were 1.2 times this value. Two ms after an initial pulse from the digitimer triggered the oscilloscope, another pulse triggered the electrical stimulator. The mechanical stimulator was triggered 200ms later. This timing sequence

ensured that the afferent fibre was stimulated electrically before a mechanical stimulus was applied to the touch dome (Fig. 2.5).

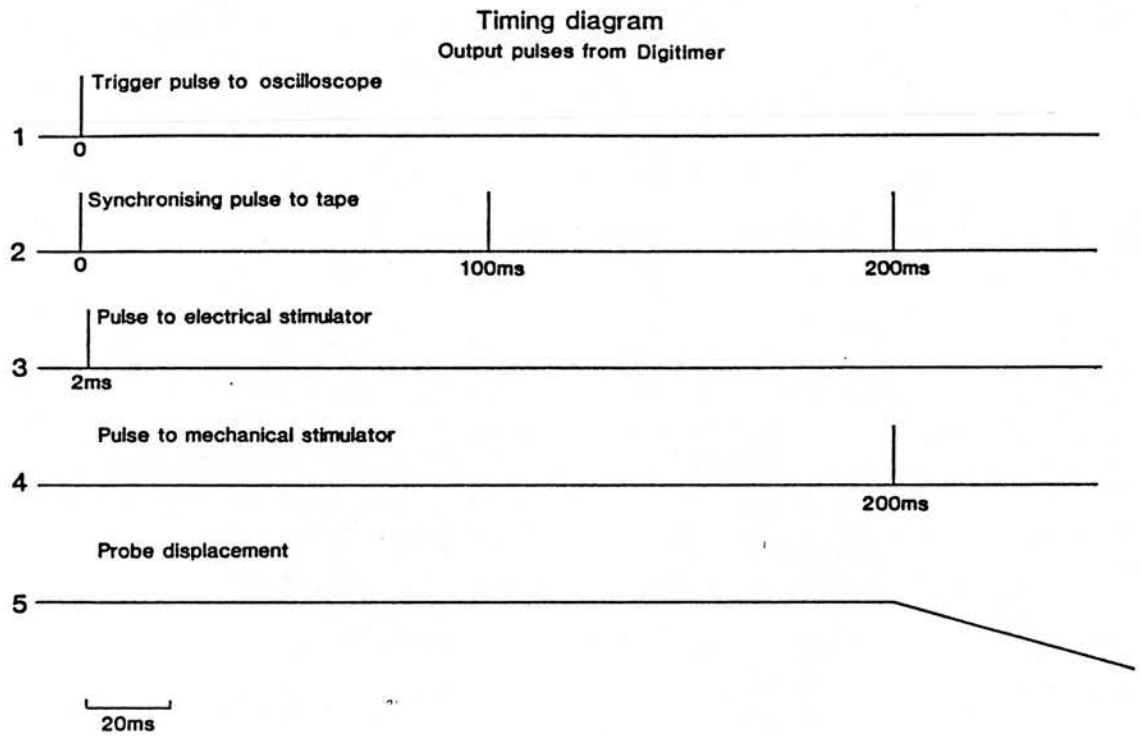
2.7 GENERAL PREPARATION FOR IN VITRO RAT EXPERIMENTS

Albino-Wistar rats (300-420g) were anaesthetised by an intra-peritoneal injection of 25% (w/v) solution of urethane in 0.9% (w/v) saline (0.7ml/kg). Supplementary doses were given as required via the external jugular vein.

The trachea was cannulated to maintain a patent airway. The animal was placed on its ventral surface on a thermostatically controlled electric blanket and the skin of the gluteal region shaved. A midline dorsal incision ~50mm long was made in the lumbar region. The skin flaps were reflected to expose the 4th and 5th dorsal cutaneous nerves on either side of the midline. The superficial fascia was removed and a black thread tied around one nerve at its point of entry into the para-spinal muscles. The nerve was transected proximal to the ligature. With the aid of the dissecting microscope and holding the ligature, the nerve was carefully dissected free from the surrounding tissue towards its point of entry into the skin. After marking the area of skin to be removed, generally 10mm x 20mm, it was freed from the underlying fascia and cut out. The skin patch and its attached nerve were then transferred to the organ bath. Gauze soaked in saline was placed over the incision and further

FIGURE 2.5

This diagram illustrates the temporal sequence of output pulses from the Digitimer.


Timing diagram
Output pulses from Digitimer
Trigger pulse to oscilloscope
1
0
Synchronising pulse to tape
2
0
100ms
200ms
Pulse to electrical stimulator
3
2ms
Pulse to mechanical stimulator
4
200ms
Probe displacement
5
20ms

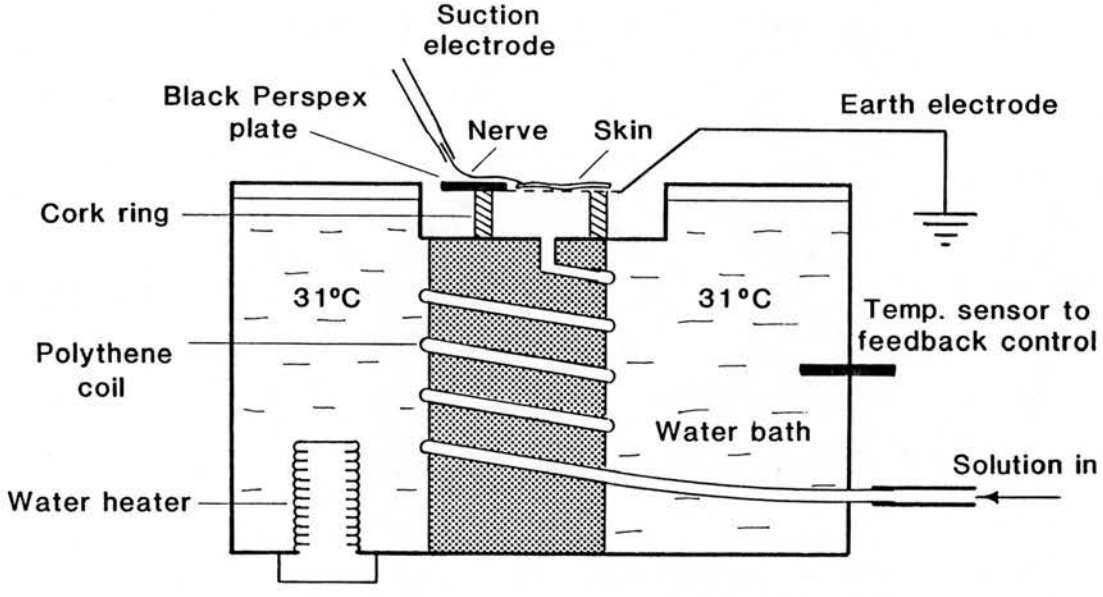
preparations removed as required. A maximum of 5 functional preparations could be removed from any one animal.

The skin-nerve preparation was then transferred to the superfusion chamber (Fig 2.6). The skin was placed epidermis uppermost on a flat stainless steel mesh disc (26mm diameter) located over a well in the superfusion chamber. The skin was stretched to its original size and held in place with entomological pins. The nerve was placed on a black perspex plate which was attached to the stainless steel platform.

The preparation was superfused with a physiological salt solution whose composition was initially identical to one used to store skin grafts minus any amino acids (Hurst, 1984). It evolved into the following composition after it was found it did not reflect rat plasma calcium and magnesium concentrations accurately enough : NaCl,116; KCl,5.4; $MgCl_2$,1.2; $CaCl_2$,2.5; dextrose, 5.6; NaH_2PO_4,1.0; $NaHCO_3$,26.0 (mM) and bubbled with carbogen (95% O_2 and 5% CO_2). The superfusing solution was heated to 31^{o}C by passing it through a coil of polythene tubing situated in a thermostatically controlled water bath. The solution flowed over the preparation at a rate of 10ml/minute. The superfusion chamber was fitted with an inlet port located beneath the mesh platform so that the incoming solution came into direct contact with the corium side of the skin. Excess solution escaped from the chamber by overflow.

FIGURE 2.6

Illustrated is a schematic diagram of the superfusion chamber for the isolated rat skin-nerve preparation. The skin was placed on a stainless steel mesh disc and pinned out to its original size. The preparation was superfused with a physiological salt solution heated to 31^{o}C by passing it through a thermostatically controlled water bath. Excess solution escaped by overflow.


Suction
electrode
Black Perspex
plate
Nerve
Skin
Earth electrode
Cork ring
31°C
31°C
Temp. sensor to
feedback control
Polythene
coil
Water bath
Solution in
Water heater

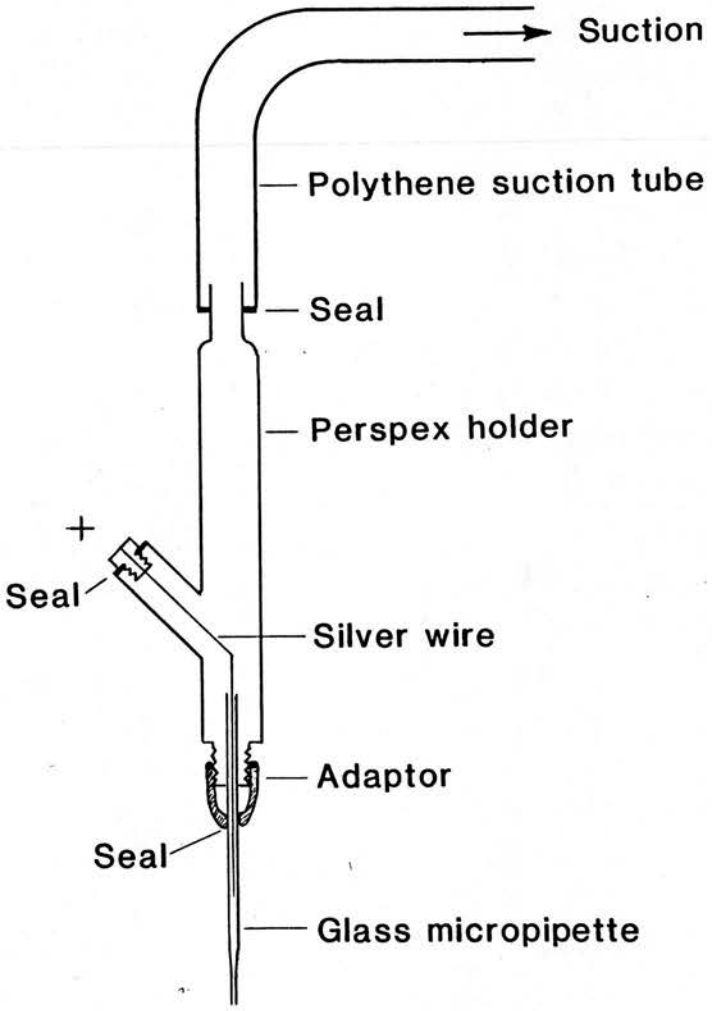
In later experiments the bicarbonate buffer system was replaced by HEPES (10mM) and the solution adjusted to pH 7.4 prior to use. This was necessary because some of the chemicals under investigation formed an insoluble precipitate with the HCO_3^- ion in the original buffering system.

Electrical activity in the nerve was recorded via a suction electrode (Fig.2.7). A glass micropipette was pulled from 1mm glass tubing and rapidly tapered. An Ag/AgCl wire was passed into the micropipette before it was mounted on a perspex holder held in a micromanipulator. The tip of the micropipette was broken with forceps under the microscope, giving an internal diameter similar to the outside diameter of the nerve after its epineurium had been removed. The electrode tip was brought very close to the cut end of the nerve which was sucked into the pipette by creating a negative pressure with a 1ml syringe attached to the polythene suction tube. A blob of silicone grease on a needle was placed close to the electrode tip and further negative pressure applied. A small amount of grease went into the space between the nerve and the internal surface of the pipette thus creating a good seal with a higher resistance. This was essential in obtaining a satisfactory signal to noise ratio.

Signals from the suction electrode were fed into the positive terminal of the preamplifier. A platinum wire was attached to the stainless steel mesh platform and this

FIGURE 2.7

This diagram illustrates the suction electrode used for recording electrical signals in the afferent fibre. The nerve was gently sucked into the electrode using a syringe connected to PVC tubing. Silicone grease was sucked into the space between the glass micropipette and the nerve, creating a good seal. Electrical contact between the nerve and the silver wire was provided by the superfusate sucked into the electrode with the nerve. Electrical signals were passed from the electrode into the preamplifier head stage.


Suction
Polythene suction tube
Seal
Perspex holder
+
Seal
Silver wire
Adaptor
Seal
Glass micropipette

acted as an earth electrode (Fig. 2.7). Signal recording and mechanical stimulation were carried out as described above.

Stimulus duration and inter-stimulus intervals were chosen to suit the time course of action of the drug under investigation; in each case the stimulus duration was 5 seconds and inter-stimulus interval 25 seconds. These stimuli were given twice per minute for a total time of 10 minutes (i.e. 20 stimulations). In every case five control stimulations were obtained before switching from the standard superfusing solution to one containing the appropriate quantity of drug.

The calcium channel blockers, cadmium chloride and verapamil hydrochloride, were given to complement the results observed in the cat models described above. After five stimulations the control solution was replaced by one containing cadmium chloride (0.05, 0.1, 0.5 or 1.0mM) or verapamil hydrochloride (20, 50 or 100uM). The effect of magnesium (5, 10 and 15mM) was also tested.

Opioid drugs were used to investigate the possibility that they might be involved in the transduction process in SA1 mechanoreceptors. After obtaining five control stimulations the control solution was replaced by one containing the opiate antagonist, naloxone (50uM). This concentration was chosen because it corresponds to the concentration found to be active in the in vivo rat model (1.0mg/kg). The opiate agonist, met-enkephalin, was also tested in this model (0.1, 1.0, 5.0

or 10.0uM), both alone and in the presence of naloxone.

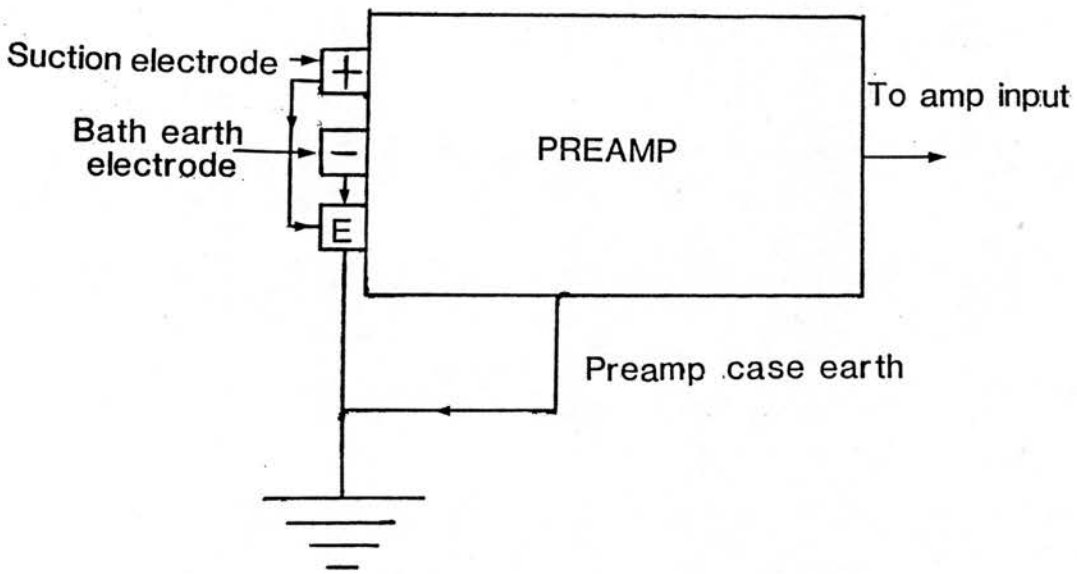
2.8 DATA ANALYSIS.

The effect of the drugs on the response of the SA1 mechanoreceptors to mechanical stimulation was assessed in two ways: 1) by comparing the number of spikes per second in response to successive stimulations under control conditions with those obtained in the presence of a drug; 2) by comparing the distribution of inter-spike intervals (ISIs) under control conditions with that when a drug is present.

Action potentials recorded on tape were passed through a spike discriminator (Digitimer Neurolog system) and analysed on a Cromemco System 3 microcomputer. For analysis of type 1) above data was collected, stored and displayed as the impulse frequency over selected time intervals which were variable from 200 to 4000ms using program RATE1 (Short,AD., unpublished). This data was then analysed using RATSTAT2 (McConnell,G., unpublished) which gave the average impulses per second over a given time selected from the histogram display produced by RATE1. The average number of events/second were measured over selected time periods corresponding to individual mechanical stimulations. These values were then subdivided into two parts, the dynamic and static components of the response (Tapper 1965; Iggo and Muir 1969). The dynamic component is defined as the first 200ms of the response and the static component the last 8s of stimuli of 10s

FIGURE 2.8

Illustrated is the recording arrangement for the isolated rat skin-nerve preparation. The output from the suction electrode was fed into the positive terminal of the preamplifier. The bath earth electrode was attached to the stainless steel platform and its output was fed into the negative input terminal of the preamplifier. These connections and the preamplifier case earth were fed into a common earth point.


Suction electrode
+
Bath earth electrode
−
E
PREAMP
To amp input
Preamp case earth

total duration and 3.6s of stimuli of 5s total duration. The data so obtained was normalized by calling the response to the first mechanical stimulation 100% and scaling the others accordingly. This allowed comparison of responses obtained in different experiments even though the responses of individual receptors were different.

For analysis of type 2) above, data was collected using program GSPIKE2 and analysed using GDISP2 (McConnell,G., unpublished). The ISIs were measured and stored during periods of mechanical stimulation using a bin width of 1ms. The data was displayed as successive interval lengths versus interval number. To facilitate analysis the trigger pulse preceding each mechanical stimulation was also displayed. This enabled the sampling time for ISIs within each mechanical stimulation to be accurately determined. The sampling period chosen corresponded to the static phase of individual mechanical stimulations.

2.9 STATISTICAL ANALYSIS OF DATA

In analysis type 1) above, an average value was calculated for each individual mechanical stimulation. Control values were compared with drug treatments and the level of significance between results tested using the Student's "t"-test.

The mean number of impulses per second was plotted against stimulus number and linear regression analysis

applied to the data under control conditions and drug treatments. The regression coefficient obtained for drug treatments was tested against that under control conditions and the difference between the two tested for significance using the Student's "t"-test.

In the analysis of type 2) above, a histogram of ISI distribution for mechanical stimulations 1, 5, 10, 15 and 19 was produced. This distribution was truncated at the maximum frequency of the histogram and an exponential of the form $f(t)=e^{-lt}$ (where l is the reciprocal of the mean ISI of the truncated distribution fitted to it. The goodness of fit of the histogram to the curve was tested using the chi squared (X^2) test.

CHAPTER 3

OPIOID EXPERIMENTS.

OPIOID EXPERIMENTS

3.1 INTRODUCTION

Met-enkephalin-like immunoreactivity has been demonstrated at the light microscope level in rodent Merkel cells (Hartschuh et al 1979) with the strongest immunoreaction occurring in the region of highest granule density. These observations led to speculation that the met-enkephalin-like substance could be a transmitter. A fundamental test of this hypothesis would be the observation of the effect of an opiate antagonist such as naloxone on the response of touch domes to controlled mechanical stimulation. The SA1-like responses of feline sinus hair follicles to a standardised mechanical stimulation after intra-venous naloxone did not change even at doses where the drug would act non-specifically (Gottschaldt and Vahle-Hinz 1982). A subsequent study showed that met-enkephalin-like immunoreactivity was peculiar to rodent Merkel cells (Hartschuh, Weihe, Yanaihara and Reinecke, 1983), therefore this negative result in the cat was to be expected. Intra-venous administration of naloxone in doses up to 10mg/kg in rats had no obvious effect on SA1 mechanoreceptors stimulated with von Frey hairs (Handwerker, personal communication). Since the stimulus applied was purely qualitative it would be difficult to draw firm conclusions from this result. A satisfactory test of the hypothesis that the met-enkephalin-like substance is a transmitter in SA1 mechanoreceptors has therefore not been carried out. The

aim of the experiments in this chapter was to establish whether opioid drugs affect the response of SA1 mechanoreceptors to controlled mechanical stimulation.

3.1 EXPERIMENTAL PROCEDURES

Initially, the effect of naloxone (0.1, 0.5 and 1.0mg/kg) on whole animals was observed as described in chapter 2.1. Mechanical stimuli were given once per minute, with a stimulus duration of 10s. Either saline or naloxone were given intra-venously after the 5th mechanical stimulus. However, in these experiments it was impossible to be sure that the effects of naloxone were exerted on the SA1 mechanoreceptors rather than the surrounding tissues. For this reason and those discussed in chapter 1.6, I developed an isolated rat skin-nerve preparation which was superfused with a solution whose composition was under control. Experiments of this type were carried out as described in chapter 2.7. Mechanical stimuli were given twice per minute with a stimulus duration of 5 s. The effect of 50uM naloxone was observed. This concentration of naloxone was chosen because it corresponds to the dose which was effective <u>in vivo.</u> The effects of met-enkephalin alone (0.1, 1.0, 5.0 and 10uM) and in the presence of naloxone were also investigated. Data analysis was carried out as described in chapter 2.8 and 2.9.

3.3 RESULTS

The effect of naloxone on SA1 mechanoreceptors.

In the in vivo rat model, the response of the touch domes to mechanical stimulation after control injections of saline (0.9%, 0.3ml, i.v.) were compared to the response of the SA1 mechanoreceptors to mechanical stimulation alone (Table 3.1). Although there is a slight increase in the responses, this is not statistically significant. Naloxone caused a dose dependent increase in the number of impulses per mechanical stimulation compared to control responses (Table 3.2). The smallest dose used (0.1mg/kg) had no effect on the whole response whereas the effect became significant 13 and 6 mins after administration of 0.5 and 1.0mg/kg naloxone respectively ($P < 0.05$, student's t-test). The effect of naloxone was more pronounced on the static component of the response (Table 3.4) where all three doses were effective. The action of naloxone was less pronounced on the dynamic component of the response (Table 3.3). These effects did not appear to be caused by a change in either arterial blood pressure or pH balance of the animal since neither parameter was affected after naloxone administration (Arterial blood pressure = 108 ± 14, 123 ± 15, 117 ± 11 and 111 ± 15 mmHg for controls, 0.1mg/kg, 0.5mg/kg and 1.0mg/kg naloxone respectively; pH = 7.41 ± 0.1 and 7.41 ± 0.06 for control and 1.0mg/kg naloxone respectively $P > 0.05$, Student's t-test).

A comparison of the slopes of the graphs showing how the response changes between the 5th and 20th stimulation (Fig. 3.1) showed that at all doses of naloxone the slope

TABLE 3.1

The effect of saline on the response of SA1 mechanoreceptors in the *in vivo* rat preparation.

STIMULUS NUMBER	CONTROL (n=11) ($10-63 cs^{-1}$)	SALINE (n=4) ($17-27 cs^{-1}$)
1	100	100
2	96±3.4	98±2.6
3	96±2.4	98±2.3
4	96±3.6	93±7.6
5	91±2.5	96±4.9
6	93±1.9	92±3.6
7	89±1.8	93±3.6
8	88±2.4	88±5.6
9	86±2.9	93±4.7
10	88±1.7	90±4.7
11	85±2.0	96±6.8
12	83±2.8	92±6.4
13	84±3.0	89±6.4
14	81±3.1	86±5.0
15	80±3.8	85±7.8
16	79±2.6	80±12.2
17	77±1.9	83±11.9
18	73±3.0	81±11.6
19	72±1.5	82±15.1
20	71±2.4	80±5.0

(MEAN ± SE)

n= number of experiments

10s Stimuli were applied at 60s intervals.

Raw data given as counts per second (cs^{-1}).

TABLE 3.2

The effect of naloxone on the whole response of SA1 mechanoreceptors in the *in vivo* rat preparation.

STIMULUS NUMBER	CONTROL (n=11) 10-63 cs^{-1}	0.1mg/kg (n=7) 28-62 cs^{-1}	0.5mg/kg (n=5) 26-52 cs^{-1}	1.0mg/kg (n=11) 7-58 cs^{-1}
1	100	100	100	100
2	96±3.4	102±2.4	105±2.9	97±0.9
3	96±2.4	102±4.8	96±1.8	97±1.3
4	96±3.6	97±2.7	96±2.4	95±3.6
5	91±2.5	90±5.3	93±3.3	98±4.1
6	93±1.9	92±4.3	95±5.5	96±3.6
7	89±1.8	88±3.0	92±3.7	99±4.0*
8	88±2.4	93±5.6	92±5.3	96±4.2
9	86±2.9	96±7.9	91±4.0	97±4.9
10	88±1.7	98±7.8	91±5.1	102±6.7
11	85±2.0	97±9.5	90±5.2	108±8.6*
12	83±2.8	96±9.0	89±5.9	105±8.7*
13	84±3.0	91±7.8	89±6.2	107±9.2*
14	81±3.1	88±7.8	88±5.9	109±10.2*
15	80±3.8	90±8.0	87±5.9	107±8.9**
16	79±2.6	92±9.1	85±5.5	104±8.9*
17	77±1.9	88±7.7	89±6.8	100±6.5**
18	73±3.0	91±9.0	89±7.4*	97±7.2**
19	72±1.5	86±6.7	85±8.5	102±7.2***
20	71±2.4	80±6.6	82±7.3	99±6.8**

(MEAN ± SE)

n= number of experiments; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

10 s Stimuli were applied at 60s intervals

Raw data given as counts per second (cs^{-1}).

TABLE 3.3

The effect of naloxone on the dynamic response of SA1 mechanoreceptors in the *in vivo* rat preparation.

STIMULUS NUMBER	CONTROL (n=11) 60-220cs^{-1}	0.1mg/kg (n=7) 105-165cs^{-1}	0.5mg/kg (n=5) 80-220cs^{-1}	1.0mg/kg (n=11) 40-235cs^{-1}
1	100	100	100	100
2	91±4.1	100±1.5	105±1.9	99±3.6
3	85±6.3	98±2.4	98±1.6	97±4.9
4	92±4.7	88±5.0	99±4.9	99±6.0
5	83±6.0	82±4.5	95±6.1	102±6.8
6	89±5.5	81±3.6	97±4.2	103±7.4
7	90±5.7	81±5.8	97±5.6	99±6.3
8	90±2.8	91±7.0	91±1.7	99±6.9
9	86±2.8	87±9.6	95±4.0	94±5.7
10	75±2.5	87±5.3	93±5.9*	102±8.7*
11	84±3.9	85±6.4	89±9.5	104±11.4
12	86±8.5	85±6.4	90±6.7	101±8.6
13	77±5.0	82±6.0	83±3.7	100±10.5
14	76±5.2	82±6.3	80±4.7	114±12.7*
15	76±6.2	84±4.4	79±5.2	100±8.9*
16	70±4.1	79±4.3	87±7.7	98±9.5*
17	77±5.1	77±5.1	92±5.3	104±8.4*
18	79±4.9	75±7.2	88±7.6	104±8.5*
19	87±6.2	80±5.7	92±5.8	100±5.9
20	83±2.7	67±13.6	91±5.2	92±8.0

(MEAN ± SE).

n= number of experiments; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

10s Stimuli were applied at 60s intervals.

Raw data given as counts per second (cs^{-1}).

TABLE 3.4

The effect of naloxone on the static response of SA1 mechanoreceptors in the *in vivo* rat preparation.

STIMULUS NUMBER	CONTROL (n=11) 11-48 cs^{-1}	0.1mg/kg (n=7) 32-74 cs^{-1}	0.5mg/kg (n=5) 18-89 cs^{-1}	1.0mg/kg (n=11) 74-68 cs^{-1}
1	100	100	100	100
2	105±4.3	100±5.0	105±2.5	99±2.0
3	98±3.2	102±5.6	98±3.4	99±3.2
4	98±3.4	95±4.5	99±4.4	97±5.8
5	93±3.5	95±4.0	96±3.8	98±6.1
6	94±3.1	91±5.5	96±6.8	96±5.0
7	89±2.3	90±4.7	93±3.8	99±5.1
8	90±2.9	96±7.5	95±5.8	96±5.1
9	87±3.2	94±8.0	90±4.1	101±6.2
10	91±3.8	98±8.4	92±5.8	102±7.4
11	89±3.3	100±10.0	93±5.8	109±8.2*
12	81±3.4	98±9.9*	91±7.3	101±4.9*
13	84±3.2	91±8.2	88±5.1	105±5.2**
14	84±3.1	90±8.2	88±6.0	102±5.6*
15	79±3.9	92±9.1	87±5.8	100±4.7*
16	79±3.3	95±10.7	87±5.9	99±4.6*
17	76±2.7	92±8.9	91±6.8*	100±4.8**
18	75±1.8	96±10.5*	90±6.7*	98±5.1**
19	72±2.8	90±7.6*	92±8.2*	118±11.4***
20	73±3.4	93±10.2	91±7.2*	118±9.4***

(MEAN ± SE).

n= number of experiments; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

10s Stimuli were applied at 60s intervals.

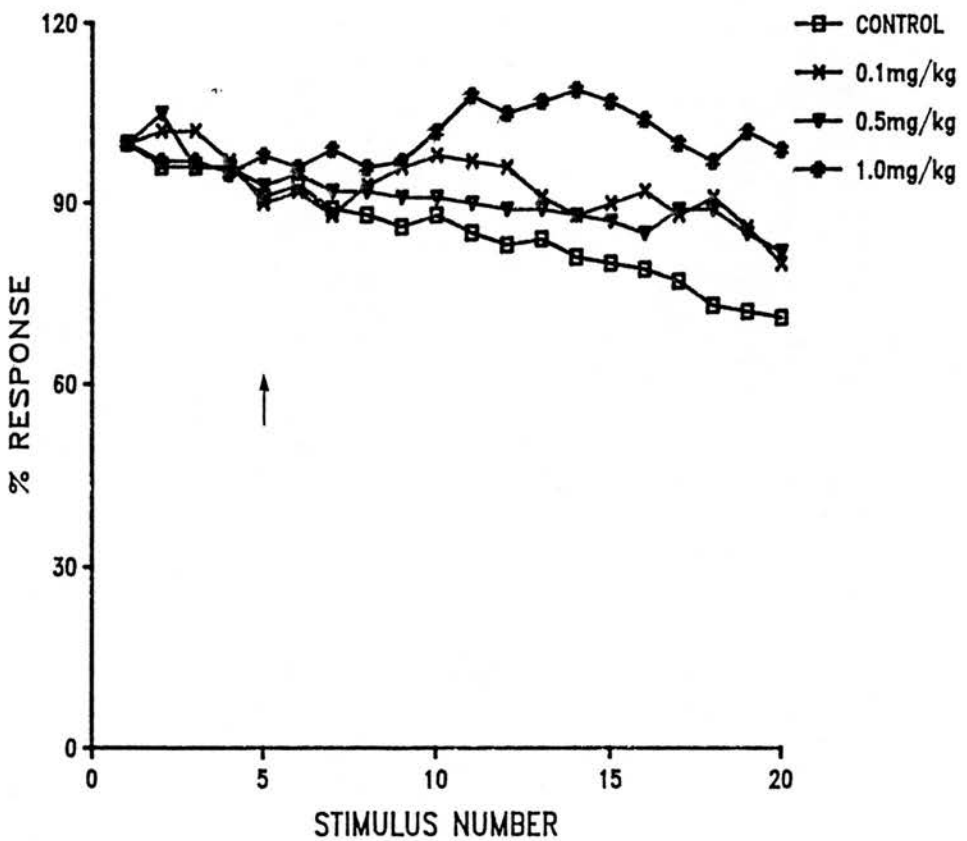
Raw data given as counts per second (cs^{-1}).

FIGURE 3.1

This graph illustrates the normalized response of touch domes in the *in vivo* rat preparation after the intravenous administration of naloxone (0.1, 0.5 or 1.0mg/kg).

The stimulus duration was 10s and stimuli were applied at 60s intervals. 5 stimuli were applied before drug administration (arrow).

Naloxone caused a dose dependent enhancement of the response to mechanical stimulation.


120
90
60
30
0
% RESPONSE
0
5
10
15
20
STIMULUS NUMBER
CONTROL
0.1mg/kg
0.5mg/kg
1.0mg/kg

was significantly higher than the control (-1.39 ± 0.08, -0.52 ± 0.22, -0.62 ± 0.08, 0.27 ± 0.24 for control, 0.1, 0.5 and 1.0mg/kg naloxone respectively; mean + sem, $P < 0.001$, students t-test).

The distribution of inter spike intervals (ISIs) during the static (adapted) component of the response was also examined. The control responses' ISI distributions did not differ significantly from an exponential distribution (Table 3.5). With one exception for every dose tested, the ISI distributions in the presence of naloxone were also exponential (Table 3.5). In those instances where there were too few intervals to test in one individual response, the trend in responses was tested for stationarity. However, in all cases this differed significantly from perfect stationarity (i.e. line of zero slope), ($P > 0.05$, student's t-test).

A typical response recorded from the isolated rat skin-nerve preparation is shown in Fig. 3.2; a typical response recorded from the more conventional *in vivo* preparation is shown for comparison. This figure illustrates that recordings obtained from the novel *in vitro* preparation are at least as satisfactory as those obtained from the other preparations used.

In the isolated rat skin-nerve preparation, the control responses appear to be more stable than those in the *in vivo* preparation i.e. do not show the same tendency to decline with increasing stimulus number. In the intact preparation, the response decreased significantly more

TABLE 3.5

Analysis of the inter-spike interval distribution in control and naloxone experiments in the *in vivo* rat preparation.

EXPERIMENT TYPE	STIMULUS NUMBER 1	5	10	15	19
CONTROLS					
21118521	+	+	+	+	+
02128521	+	0	0	0	0
04128521	+	+	+	+	+
15018621	+	+	+	+	+
22018621	+	+	+	+	+
28018623	+	+	+	+	+
10028621	+	+	+	+	+
11028621	+	+	+	+	+
17028621	+	+	+	+	+
25018622	+	+	+	+	+
28028623	+	+	+	+	+
0.1mg/kg					
19038621	+	+	+	+	+
26038621	+	+	+	+	0.05 >P >0.02
10048621	+	+	+	+	+
12048621	+	+	+	+	+
19048621	+	+	+	+	+
22048622	+	+	+	+	+
23048621	+	+	+	+	0
0.5mg/kg					
22048621	+	+	+	+	+
13058621	+	+	+	+	+
14058622	+	+	0.02>P>0.01	0.02>P>0.01	0
27058621	+	+	+	+	+
05068721	+	+	+	+	+
1.0mg/kg					
02128522	0	0	0	0	0
04128522	+	+	+	+	+
22018622	+	+	+	+	0.02>P>0.01
25028621	+	+	+	+	+
13038622	+	+	+	+	+
14038621	+	+	+	+	+
18038622	+	+	+	+	+
12048622	+	+	+	+	+
18048621	+	+	+	+	+
19048623	+	+	0.05>P>0.02	+	+
20048622	+	+	+	+	+

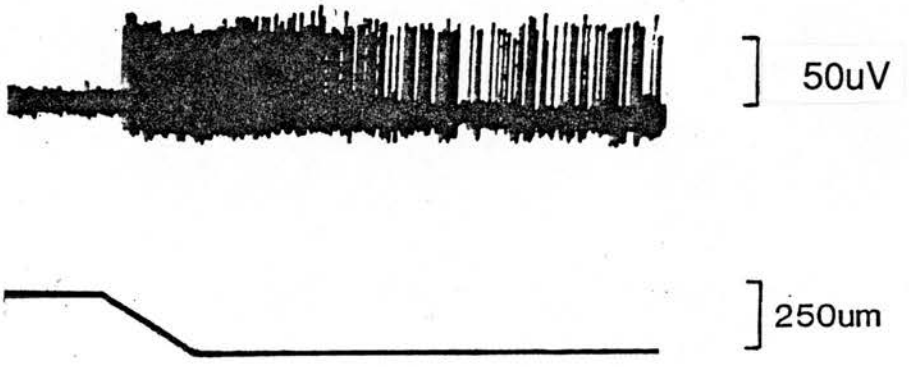
+ = exponential distribution; 0 = too few classes to test;

Other entries indicate that the ISI distribution is not exponential

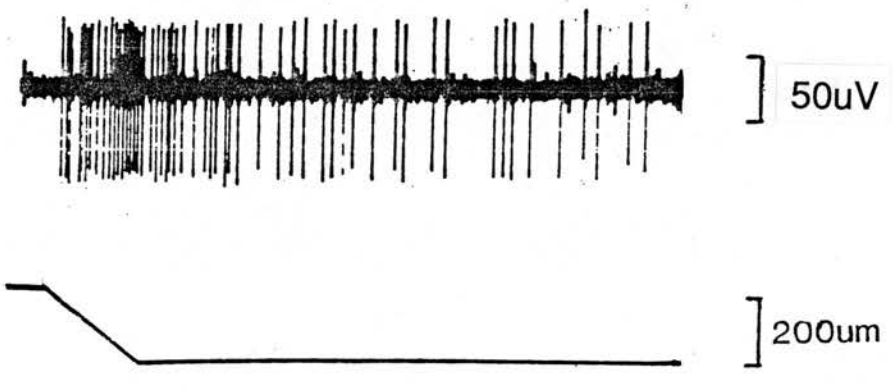
FIGURE 3.2

Illustrated are oscilloscope traces comparing control responses from the *in vivo* rat preparation and the *in vitro* rat skin-nerve preparation. This demonstrates that the records obtained from the novel *in vitro* preparation are of similar quality to those obtained in the other preparations used. The mechanical stimulation is shown as a downward deflection in the lower half of each record.

Invivo rat preparation



In vitro rat preparation



1
0.2ms

than in the isolated preparation (-29.0 ± 2.0% and -7.0 ± 1.7% ; mean± se for the intact and isolated preparations respectively; $P < 0.05$, student's t-test).

In the in vitro preparation naloxone (50uM) had no effect on either the whole, the dynamic or the static components of the responses of the SA1 mechanoreceptors to mechanical stimulation (Tables 3.6, 3.7 and 3.8 respectively). Nor was there any difference in the slopes of the graphs showing how the response changed from the 5th to the 20th stimulation (Fig. 3.3), (-1.31 ± 0.19, -1.56 ± 0.11, control and 50uM naloxone respectively; mean ± sem).

In two of the eight control experiments there were insufficient intervals to test the ISI distributions. With these exceptions, the ISIs of the adapted discharge of all the other control experiments had an exponential distribution (Table 3.9). In all experiments except those where there were too few intervals to test, i.e. approximately half, the ISIs distribution in the presence of naloxone were also exponential (Table 3.9).

The effect of met-enkephalin on the response of SA1 mechanoreceptors in the isolated rat skin-nerve preparation.

Met-enkephalin caused a dose-dependent decline in the response of SA1 mechanoreceptors to a standard mechanical stimulation (Table 3.10). The effect became established ~2.5 minutes after the administration of the smaller doses

TABLE 3.6

The effect of naloxone on the whole response in the isolated rat skin-nerve preparation.

STIMULUS NUMBER	CONTROL (n=7) 28-88cs^{-1}	NALOXONE (50uM) (n=7) 21-46cs^{-1}
1	100	100
2	106±2.4	106±4.0
3	102±5.4	102±3.2
4	101±5.5	102±2.8
5	106±5.0	98±2.1
6	106±4.2	103±3.7
7	110±6.8	96±1.3
8	104±4.8	94±5.9
9	102±5.3	95±5.9
10	95±2.4	89±6.9
11	101±4.6	88±7.1
12	101±5.9	88±7.3
13	99±4.5	88±7.6
14	97±4.6	88±7.2
15	96±4.4	84±5.0
16	93±4.1	81±4.6
17	86±3.7	79±4.9
18	91±4.7	80±4.7
19	89±3.6	80±4.2
20	90±5.7	76±4.0

(MEAN ± SE).

n= number of experiments.

5s Stimuli were applied at 30s intervals.

Raw data given as counts per second (cs^{-1}).

TABLE 3.7

The effect of naloxone on the dynamic response of SA1 mechanoreceptors in the isolated rat skin-nerve preparation.

STIMULUS NUMBER	CONTROL (n=7) 95-230cs^{-1}	NALOXONE (50uM) (n=7) 75-130cs^{-1}
1	100	100
2	93±3.9	108±5.1*
3	93±3.5	103±6.7
4	99±6.4	104±7.0
5	93±3.5	101±6.1
6	97±2.0	98±7.1
7	92±2.8	93±5.0
8	93±2.8	97±9.1
9	96±3.9	101±11.3
10	93±3.6	96±10.4
11	96±6.8	95±9.6
12	96±3.1	91±10.1
13	102±6.1	91±10.4
14	97±4.8	90±4.2
15	90±6.2	94±3.1
16	85±3.2	87±4.2
17	86±6.6	84±5.4
18	89±7.8	86±5.2
19	90±7.1	89±5.6
20	94±7.0	87±7.2

(MEAN ± SE)

n= number of experiments; 5 Stimuli were applied at 30s intervals.

Raw data given as counts per second (cs^{-1}).

TABLE 3.8

The effect of naloxone on the static response of SA1 mechnoreceptors in the isolated rat skin-nerve preparation.

STIMULUS NUMBER	CONTROL (n=7) 21-75cs^{-1}	NALOXONE (50uM) (n=7) 19-43cs^{-1}
1	100	100
2	101±5.7	110±5.0
3	102±4.8	106±3.2
4	98±7.0	99±4.2
5	105±7.8	98±1.8
6	105±6.7	104±4.0
7	110±10.0	96±3.8
8	100±7.6	93±7.1
9	100±8.6	94±4.7
10	96±6.4	92±6.4
11	100±5.1	86±6.7
12	106±6.7	88±6.4
13	104±6.3	92±6.5
14	99±5.5	88±6.5
15	104±5.4	76±4.2
16	96±5.9	82±5.5
17	95±5.3	81±3.9
18	98±5.8	83±4.8
19	94±7.3	77±3.2
20	92±7.0	78±4.0

(MEAN ± SE)

n=number of experiments

5s Stimuli were applied at 30s intervals.

Raw data given as counts per second (cs^{-1}).

FIGURE 3.3

This graph illustrates the normalized responses of SA1 mechanoreceptors to mechanical stimulation in the isolated rat skin-nerve preparation. Control experiments and experiments in which the preparation was superfused with a solution containing 50uM naloxone are shown. Stimuli were applied at 30s intervals and the stimulus duration was 5s. 5 stimuli were applied before superfusion with the solution containing naloxone (arrow). Naloxone had no significant effect on the SA1 mechanoreceptors in this preparation.

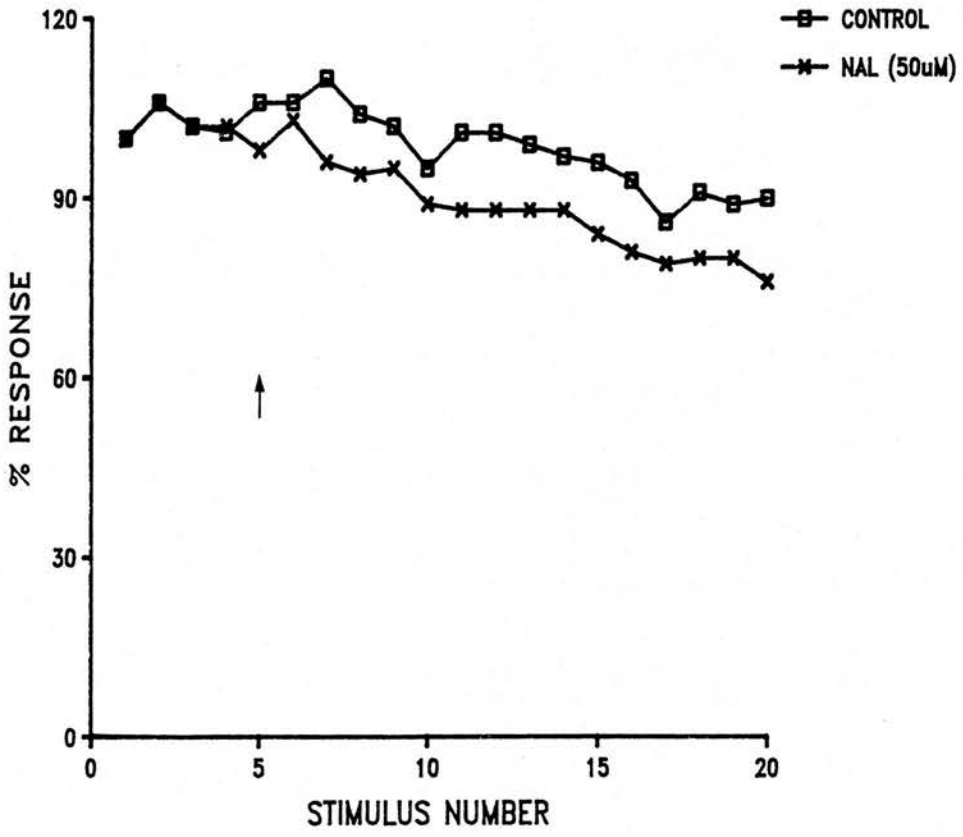

CONTROL
NAL (50uM)
120
90
60
30
0
% RESPONSE
0
5
10
15
20
STIMULUS NUMBER

TABLE 3.9

Analysis of the inter-spike interval distribution in control and naloxone experiments in the isolated rat skin-nerve preparation.

EXPERIMENT TYPE	STIMULUS NUMBER 1	5	10	15	19
27078721	+	+	0	0	0
04088721	+	+	+	+	+
28088722	+	+	+	0	0
01098721	+	+	+	+	+
01098724	+	+	+	+	+
01098725	+	+	+	+	+
02098726	+	+	+	+	+
03098727	+	+	+	+	+
NALOXONE (50uM)					
19068722	+	+	+	+	+
04088723	+	+	+	0	0
05088721	+	+	+	+	+
05088722	+	+	0	0	0
04098722	+	+	0	0	0
04098723	0	0	0	0	0
04098725	+	+	+	+	0

+ = exponential distribution; 0 = too few classes to test

TABLE 3.10

The effect of met-enkephalin on the whole response of SA1 mechanoreceptors in the isolated rat skin-nerve preparation.

STIMULUS NUMBER	CONTROL (n=7) 28-88cs^{-1}	0.1uM (n=4) 14-53cs^{-1}	1.0uM (n=5) 23-84cs^{-1}	5.0uM (n=5) 20-54cs^{-1}	10uM (n=6) 18-46cs^{-1}
1	100	100	100	100	100
2	106±2.4	103±5.2	104±2.6	101±2.0	95±4.0
3	102±5.4	100±7.4	98±1.9	102±4.0	94±4.5
4	101±5.5	102±6.4	92±4.4	91±4.3	97±5.6
5	106±5.0	95±5.6	94±4.7	95±2.5	94±7.3
6	106±4.2	98±1.8	91±4.7*	86±6.0*	92±7.2
7	110±6.8	88±5.3	91±4.8*	85±5.9*	82±8.5*
8	104±4.8	84±8.2	89±5.9	70±4.6**	77±9.7*
9	102±5.3	76±9.8*	86±6.3	78±3.9**	69±11.1*
10	95±2.4	71±9.0*	81±5.8*	80±4.3*	62±10.9*
11	101±4.6	66±9.3**	81±5.4*	73±4.0***	56±12.0**
12	101±5.9	67±9.0*	79±6.3*	70±3.5***	55±14.6*
13	99±4.5	62±7.9**	76±6.0*	65±4.4***	49±15.5**
14	97±4.6	66±10.2*	65±5.2***	61±4.8***	45±15.7**
15	96±4.4	64±9.1**	62±7.2**	57±7.2***	40±17.0**
16	93±4.1	62±8.1**	55±5.4***	55±8.4**	43±18.2*
17	86±3.7	58±6.8**	53±7.5**	46±8.4**	33±14.8**
18	91±4.7	57±7.7**	50±7.9	42±10.0***	33±14.7**
19	89±3.6	59±8.7**	45±8.1	37±10.4***	33±15.2**
20	90±5.7	56±8.6**	42±7.2***	32±11.0***	32±15.1**

(MEAN ± SE)

n= number of experiments; * P < 0.05; ** P < 0.01; *** P < 0.001

5s Stimuli were applied at 30s intervals.

Raw data given as counts per second (cs^{-1}).

(0.1 and 1.0uM) and ~1 minute after administration of the two higher doses. The dynamic phase of the response was less affected than the whole response, except at the highest dose (Table 3.11). 0.1uM met-enkephalin had no effect and the onset of the effects of 1.0 and 5.0uM were slower though their maximum inhibitory effects were almost identical. The effect of met-enkephalin on the static response was very similar to its effect on the whole response (Table 3.12). The differential effect of met-enkephalin on the two components of the response was shown more clearly by comparing their ID_{50} values (i.e. concentration which causes 50% inhibition of the response) : 1.37 $\pm$ 0.39 and 0.21 $\pm$ 0.11uM for the dynamic and static components respectively (mean $\pm$ S.E). The ID_{50} value for the dynamic response is significantly higher than that for the static component ($P < 0.05$, student's t-test). These results strongly suggest that the dynamic phase of the response is less sensitive to met-enkephalin than the static component.

Changes in the response between the 5th and 20th response are shown graphically in Fig. 3.4. Comparison of the slopes showed that those of met-enkephalin are significantly lower than the control and are dose dependent (-1.31 $\pm$ 0.19, -2.62 $\pm$ 0.29, -3.70 $\pm$ 0.19, -3.92 $\pm$ 0.16 and -4.36 $\pm$ 0.28 for control, 0.1, 1.0, 5.0 and 10.0uM met-enkephalin respectively; mean $\pm$ sem, student's t-test, $P < 0.001$).

The effect of met-enkephalin (0.1, 1,0 and 10.0uM)

TABLE 3.11

The effect of met-enkephalin on the dynamic response of SA1 mechanoreceptors in the isolated rat skin-nerve preparation.

STIMULUS NUMBER	CONTROL (n=7) 95-230cs^{-1}	0.1uM (n=5) 25-160cs^{-1}	1.0uM (n=5) 70-230cs^{-1}	5.0uM (n=5) 85-160cs^{-1}	10uM (n=6) 75-130cs^{-1}
1	100	100	100	100	100
2	93±3.9	100±9.9	92±4.8	101±7.4	88±3.0
3	93±3.5	100±2.2	88±5.3	101±6.5	92±8.0
4	99±6.4	103±8.6	87±5.6	90±5.7	84±6.9
5	93±3.5	94±2.6	92±6.1	94±10.7	91±4.9
6	97±2.0	104±12.2	75±7.9***	85±8.5	82±4.7*
7	92±2.8	87±9.3	77±8.0	84±6.9	77±3.2**
8	93±2.8	92±10.4	74±7.9*	75±11.9	74±5.7*
9	96±3.9	79±8.0	72±8.7*	74±8.5*	68±6.6**
10	93±3.6	80±10.5	68±6.2**	71±9.0*	61±8.3**
11	96±6.8	74±13.1	70±8.4	59±8.2**	56±9.4**
12	96±3.1	72±12.8	69±8.0**	64±8.4**	54±11.8**
13	102±6.1	79±16.8	66±6.4**	55±8.3***	46±10.6***
14	97±4.8	67±14.1	62±4.3	55±8.1***	43±10.4***
15	90±6.2	68±13.8	57±8.0**	51±10.0**	42±12.9**
16	85±3.2	75±18.0	58±7.3**	47±8.1**	39±14.6**
17	86±6.6	76±20.3	54±4.3**	47±7.0**	33±13.2**
18	89±7.8	70±15.0	55±5.8**	38±7.3***	34±15.0**
19	90±7.1	68±15.4	44±4.6***	35±7.9***	30±14.2**
20	94±7.0	83±21.3	46±4.8***	36±8.6***	31±14.0**

(MEAN ± SE)

n= number of experiments; * P < 0.05; ** P < 0.01; *** P < 0.001

5s Stimuli were applied at 30s intervals.

Raw data given as counts per second (cs^{-1}).

TABLE 3.12

The effect of met-enkephalin on the static response of SA1 mechanoreceptors in the isolated rat skin-nerve preparation.

STIMULUS NUMBER	CONTROL (n=7) 21-75 cs^{-1}	0.1uM (n=5) 12-42 cs^{-1}	1.0uM (n=5) 18-73 cs^{-1}	5.0uM (n=5) 15-45 cs^{-1}	10uM (n=6) 14-37 cs^{-1}
1	100	100	100	100	100
2	101±5.7	108±6.0	106±1.4	106±6.4	92±4.5
3	102±4.8	100±11.0	99±1.7	100±3.1	84±5.7*
4	98±7.0	105±8.8	93±4.7	88±6.7	79±8.7
5	105±7.8	97±7.0	93±5.7	89±8.9	82±7.4
6	105±6.7	102±3.1	92±3.7	84±6.9*	81±7.9*
7	110±10.0	89±4.9	90±5.1	83±7.3	76±8.8*
8	100±7.6	88±7.0	90±5.9	80±6.2	68±8.8*
9	100±8.6	76±8.4	86±5.4	76±5.3*	62±11.0*
10	96±6.4	73±8.4	82±5.0	80±6.1	57±10.0**
11	100±5.1	67±7.6**	80±5.6*	74±6.9*	51±10.8**
12	106±6.7	62±9.3**	78±5.0**	67±4.2***	52±14.1**
13	104±6.3	63±7.7*	74±6.0**	62±5.8***	44±15.9**
14	99±5.5	68±10.3*	64±5.5***	58±5.6***	39±16.4**
15	104±5.4	64±8.3**	64±9.0**	56±7.6***	38±15.9**
16	96±5.9	60±5.8**	52±6.9***	53±8.8**	42±18.4*
17	95±5.3	60±5.7**	51±10.1**	42±7.7***	31±14.0**
18	98±5.8	56±7.5**	46±8.7***	40±10.0***	30±13.6***
19	94±7.3	62±7.7*	45±9.1**	34±10.4***	30±14.1**
20	92±7.0	56±7.7**	38±9.2***	29±11.4***	31±13.7**

(MEAN ± SE)

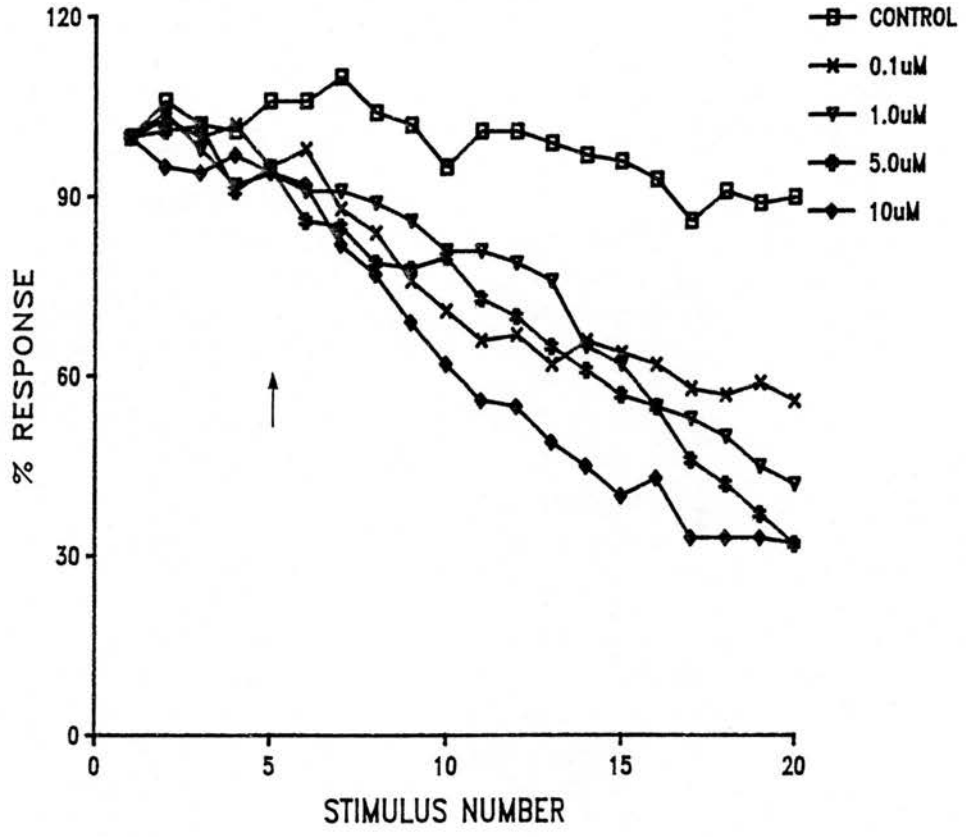
n= number of experiments; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

5s Stimuli were applied at 30s intervals

Raw data given as counts per second (cs^{-1}).

FIGURE 3.4

This graph illustrates the normalized responses of touch domes to mechanical stimulation in the isolated rat skin-nerve preparation during superfusion with a solution containing met-enkephalin (0.1, 1.0, 5.0 or 10uM). Stimuli were applied at 30s intervals and the stimulus duration was 5s. The solution containing met-enkephalin was applied after the fifth mechanical stimulus (arrow). Met-enkephalin caused a dose dependent decline in the response of the touch domes to mechanical stimulation.


120
90
60
30
0
% RESPONSE
0
5
10
15
20
STIMULUS NUMBER
CONTROL
0.1uM
1.0uM
5.0uM
10uM

was also observed in the presence of naloxone (50uM). Naloxone completely blocked the effect of met-enkephalin on the whole response (Table 3.13). There was no difference between the slope of the control response and those of met-enkephalin plus naloxone, (Fig. 3.5), (-1.31 ± 0.11, -1.04 ± 0.14, -1.85 ± 0.16 and -1.38 ± 0.16 for control, 0.1, 1.0 and 10.0uM met-enkephalin + naloxone).

In almost all experiments in the presence of met-enkephalin, there were too few intervals within one response to test the ISI distribution and an insufficient degree of stationarity to allow pooling of successive responses (Table 3.14). In those instances where there were enough intervals to test, the results obtained were not consistent but show that some ISI distributions were not exponential. In experiments where the effect of met-enkephalin was blocked by naloxone, there was also inconsistency in the nature of the ISI distribution (Table 3.14). The lack of consistency in the results obtained from these sets of experiments makes their interpretation difficult.

3.4 DISCUSSION

During the repetitive mechanical stimulation of SA1 mechanoreceptors under control conditions in the *in vivo* preparation, there was a decrease in the responsiveness of the touch domes with increasing stimulus number (-29 ± 2.0%, mean ± s.e.).This phenomenon has been described previously (Pubols, 1982b; Baumann, Hamann, Lee, Leung, 1985). The responsiveness of cutaneous mechanoreceptors is

TABLE 3.13

Comparison of the effect of naloxone with naloxone plus met-enkephalin on the whole response in the isolated rat skin-nerve preparation.

STIMULUS NUMBER	NAL (50uM) (n=7) 21-46 cs^{-1}	0.1uM ME+NAL (n=3) 22-51 cs^{-1}	1.0uM ME+NAL (n=4) 27-71 cs^{-1}	10uM ME+NAL (n=5) 28-52 cs^{-1}
1	100	100	100	100
2	106±4.0	97±3.9	101±0.9	99±2.2
3	102±3.2	103±1.7	107±5.2	99±2.2
4	102±2.8	98±5.7	101±3.8	94±1.2
5	98±2.1	93±4.0	97±2.5	95±4.1
6	103±3.7	97±4.8	95±0.7	93±5.0
7	96±1.3	96±2.6	91±3.9	88±7.0
8	94±5.9	92±3.5	90±1.7	88±5.7
9	95±5.9	87±3.2	85±2.1	84±8.1
10	89±6.9	90±3.0	84±2.0	84±6.7
11	88±7.1	92±6.3	80±2.1	80±6.7
12	88±7.3	90±4.9	75±3.2	90±5.6
13	88±7.6	86±5.8	72±1.6	79±6.2
14	88±7.2	86±4.3	74±5.4	80±7.5
15	84±5.0	84±6.4	71±2.5	77±6.5
16	81±4.6	88±11.5	71±5.5	78±6.9
17	79±4.9	81±6.7	66±3.4	75±6.3
18	80±4.7	79±6.4	71±5.9	76±6.6
19	80±4.2	84±9.6	71±4.1	71±7.1
20	76±4.0	79±9.3	66±3.5	74±6.7

(MEAN ± SE)

n= number of experiments; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

5s Stimuli were applied at 30s intervals.

Raw data given as counts per second (cs^{-1}).

FIGURE 3.5

This graph illustrates the normalized response of SA1 mechanoreceptors in the isolated rat skin-nerve preparation after superfusing with a solution containing 50uM naloxone or a solution containing 50uM naloxone plus met-enkephalin (0.1, 1.0 or 10uM). The solution containing the drugs was applied after the fifth mechanical stimulus (arrow). Stimuli were applied at 30s intervals with a stimulus duration of 5s. These results illustrate that naloxone completely antagonizes the attenuation in the response caused by met-enkephalin.

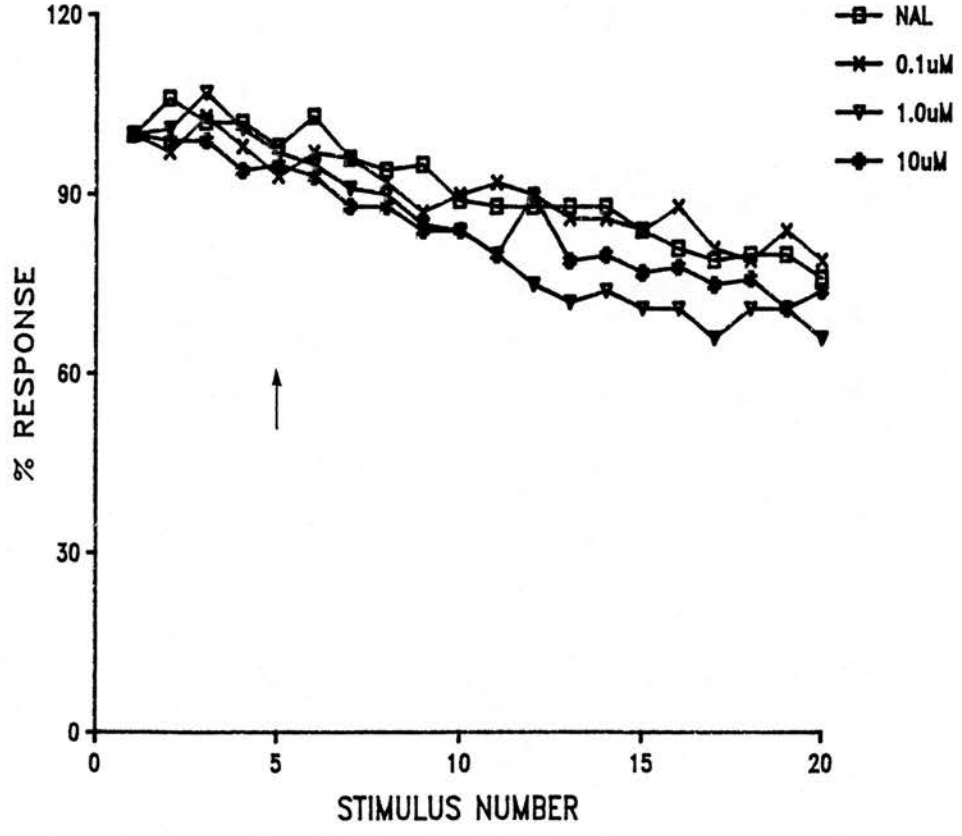

120
90
60
30
0
% RESPONSE
0
5
10
15
20
STIMULUS NUMBER
NAL
0.1uM
1.0uM
10uM

TABLE 3.14

Analysis of inter-spike interval distribution in met-enkephalin experiments in the isolated rat skin-nerve preparation.

EXPERIMENT TYPE	STIMULUS NUMBER 1	5	10	15	19
0.1uM ME					
27108721	0	0	0	0	0
27108723	+	+	0	0.05>P>0.02	0
27108725	+	+	+	0	0
27108728	+	+	+	+	0.05>P>0.02
1.0uM ME					
02098721	+	+	0.01>P>0.001	0	0
02098724	+	+	+	0	0
02098725	0	+	0	0	0
02098727	+	+	+	0.05>P>0.02	+
03098722	+	0	0	0	0
5.0uM ME					
03098724	+	+	0	0	0
03098723	+	+	+	+	0
03098724	+	+	0	0	0
03098725	+	+	0.01>P>0.001	+	0
03098726	0	0	0	0	0
10uM ME					
28088721	0	0	0	0	0
28088723	0	0	0	0	0
01098722	+	0	0	0	0
01098723	+	+	0	0	0
01098726	+	0	0	0	0
02098722	+	+	+	0	0
0.1uM ME+NAL					
27108722	+	+	+	+	+
27108726	+	+	0.02>P>0.01	0	0
27108724	0	0	0	0	0
28108723	+	+	+	+	+
1.0uM ME+NAL					
28108721	+	+	+	0	0
28108722	+	+	+	+	+
23108723	0	0	0	0	0
28108724	+	+	+	0.02>P>0.01	0
10uM ME+NAL					
11098721	0	0	0	0	0
11098722	+	+	+	+	+
11098723	+	+	0	0	0
11098724	+	+	+	+	P>0.001
11098725	+	+	0.02>P>0.01	0	0

+ = exponential distribution; 0 = too few classes to test; other entries indicate the ISI distribution is not exponential

affected by the viscoelastic properties of the skin and underlying tissues which manifest themselves in phenomena like stress relaxation, creep and creep recovery (Pubols 1982a; Baumann, Hamann, Leung 1984).It has been suggested that incomplete creep recovery is a major factor responsible for decreasing the nervous response of touch domes to repetitive stimuli (Pubols, 1982b) but this can be partly overcome by using constant force as opposed to constant displacement mechanical stimuli (Pubols, 1982a; Baumann, Hamann, Lee and Leung 1986).

In the in vitro preparation, there was a much smaller decline in response with increasing stimulus number (-7 ± 1.7%, mean ± se). This is probably due to the rigid support platform to which the skin is fixed limiting the skin creep away from the stimulus probe during repetitive constant displacement mechanical stimulation. The reduction of decreased responsiveness of touch domes with increasing stimulus number in this preparation is one of the particular advantages of the isolated preparation over the whole preparation.

Naloxone caused a dose dependent enhancement of the response to mechanical stimulation in the intact preparation (Table 3.1), the static phase being the more sensitive component of the response. Even at the highest dose, naloxone did not change the exponential distribution of ISIs (Table 3.5). From these experiments it was not clear whether naloxone was having a direct effect on the Merkel cell-neurite complex, either on the Merkel cell or

neurite membrane, or an indirect effect on the surrounding tissues. It was thought that this question may be resolved by testing the action of naloxone in the isolated preparation. However, in contrast to its effect in vivo, naloxone was without effect in vitro. There are three possible explanations for this discrepancy: 1) Naloxone cannot gain access to the Merkel cell-neurite complex. This explanation is unlikely, since other drugs were known to be effective in the isolated preparation. 2) Since naloxone increased the firing in the afferent fibre in vivo, this could be taken as evidence for tonic release of an opioid substance such as the met-enkephalin-like substance in the Merkel cell granules. The mechanism for such a tonic release may have been destroyed in the isolated preparation. 3) The action of naloxone in the intact preparation was an indirect one on surrounding tissues that were absent in the isolated preparation. Naloxone for example has been shown to increase local blood flow in the human finger (Archer, Benroubi, Pyke and Wiles 1983). If it acted in a similar manner in the intact preparation then the viscoelastic properties of the tissues underlying the skin would be changed in such a way that skin creep away from the stimulus probe would be reduced by increased blood flow through the tissues.

Until the site of action of naloxone is determined, it is impossible to distinguish between explanations 2) and 3). One possibility would be that the naloxone sensitive receptors could be found using radioligand binding studies.

Met-enkephalin caused a dose-dependent decline in the response of SA1 mechanoreceptors to controlled mechanical stimulation (Table 3.10). The dynamic component of the response was less sensitive to the effect of met-enkephalin than the static component at the lower doses (Tables 3.11 and 3.12). This result was further supported by a comparison of the ID_{50} values for the dynamic and static components which showed the former to be significantly higher than the latter. This may be partly explained by the observation that though a slowly adapting response was never observed, afferent fibres regenerating towards touch domes elicit a brief response when mechanically stimulated (Brown and Iggo 1963); perhaps this response corresponds to an insensitive part within the dynamic component of the response.

The inhibitory effect of met-enkephalin in this preparation reflects its and other opioids' actions in other areas of the mammalian nervous system (Duggan and North, 1983). Met-enkephalin *in vivo* also depresses chemosensory discharge from the cat carotid body, an organ with several morphological similarities to SA1 mechanoreceptors, (McQueen and Ribeiro, 1980; 1981a; Kirby and McQueen, 1986). How met-enkephalin inhibits the response of SA1 mechanoreceptors to mechanical stimulation remains to be established. In other systems opioids cause membrane hyperpolarization, eg at rat locus coeruleus neurones (Williams, Egan and North, 1982) possibly by increasing K^+ conductance (North and Williams, 1985) and

they depress transmitter release (Yaksh, Jessell, Gamse, Mudge and Leeman, 1980) possibly by impairing calcium entry or making intracellular calcium- evoked release less effectively. These mechanisms are discussed in more detail in chapter 5.

The effect of met-enkephalin on the distribution of ISIs is difficult to interpret because there tended to be too few intervals to test and where tests were possible the results were not consistent (Table 3.14). These problems may be overcome by increasing the stimulus duration and therefore the sampling period or the stimulus amplitude.

The effect of met-enkephalin was antagonised by the opiate antagonist naloxone (Table 3.13). The ISI distribution is, once again, difficult to interpret for the same reasons as those given in the preceding paragraph.

From these results it appears that rat SA1 mechanoreceptors possess functional opioid receptors. This gives rise to the question of the nature of the opioid receptor subclass involved. It is generally believed that there are at least three opioid receptor subtypes, designated mu, delta and kappa on the basis of their ligand specificity (Paterson, Robson and Kosterlitz, 1983). Low doses of naloxone have a greater affinity for mu receptors than the either of the other subtypes (Paterson et al 1983) which suggests the mu receptor may

not be involved in SA1 mechanoreceptors. Enkephalins have a greater affinity for delta than for mu or kappa sites but met-enkephalin is not sufficiently selective to be used for receptor classification. Relatively high doses of naloxone will affect both delta and kappa receptors as well as mu receptors (Magnan, Paterson, Tavani and Kosterlitz, 1982). Hence, there is insufficient evidence in the present results to classify the opioid receptor in rat SA1 mechanoreceptors. More selective agonists and antagonists would need to be used to resolve this question.

The existence of functional opioid receptors in rat SA1 mechanoreceptors also raises the question of the role of opioids in the transduction process. This question will be discussed in chapter 5.

CHAPTER 4

CALCIUM CHANNEL BLOCKER EXPERIMENTS.

CALCIUM CHANNEL BLOCKER EXPERIMENTS

4.1 INTRODUCTION

It is well established that extracellular calcium is required for the release of various substances including neurotransmitters from secretory cells (Rubin, 1970). It is probably a rise in intracellular ionized calcium which is the trigger for stimulus-secretion coupling since secretion occurs when calcium is introduced directly into cells (Baker and Knight, 1978) and a rise in intracellular ionized calcium associated with secretion has been directly measured in a few cells such as the squid giant synapse (Llinas, 1972) and isolated bovine medullary cells (Knight and Kesteven, 1983). In chapter 1 ultrastructural and immunocytochemical evidence was presented which suggests Merkel cells may be neurosecretory cells.

There are several structural similarities between Merkel cell-neurite complexes and hair cells of the vertebrate auditory system. The cytoplasmic processes on the apical surface of the Merkel cell may be analagous to the stereocilia, which contain actin filaments (Flock and Cheung, 1977), of hair cells. Displacement of these stereocilia is the first step in the transduction process in hair cells and it is considered likely that mechanical energy is transduced to an electrical signal through mechanically gated ionic channels, mechano-electric transducer (m-e.t) channels (Ohmori, 1987). The site of the m-e.t channel is not established. Hudspeth (1982) suggested that it existed at the tip of each stereocilia.

Angular displacement of the hair bundle about its point of insertion into the cuticle rather than absolute displacement of the hair bundle was found to be crucial for mechano-electric transduction (Ohmori, 1987) which suggests the m-e.t channel lies at the base of the stereocilia. Calcium binding sites are associated with the stereocilia of frog and guinea-pig hair cells (Moran, Carter and Asher, 1981). Ohmori (1985) demonstrated that extra-cellular calcium was required for activation of the m-e.t channel in chick vestibular hair cells. The conductance of these channels was reduced by Co^{2+} and La^{2+} (Ohmori, unpublished). D600 (methoxy-verapamil) also blocks hair cell transduction (Jorgensen, 1979). Measurement of ionic selectivities of the m-e.t channel in isolated chick hair cells demonstrated a strong selectivity for divalent cations, especially calcium (Ohmori, 1988). These cells were loaded with Fura-2 and calcium influx enhanced fluorescence intensity around the insertion of the hair bundle into the cuticle, while manganese influx quenched the fluorescence. It has been suggested that displacement of the cytoplasmic processes of Merkel cells may be the initiating step for transduction in SA1 mechanoreceptors (Iggo and Findlater, 1984).

If transmitter release is involved in the transduction process of SA1 mechanoreceptors, then interference with the entry of extra cellular calcium should prevent stimulus-secretion coupling and therefore

neural activity in the afferent fibre. The aim of the experiments in this chapter was to test the hypothesis that calcium entry was required for the normal response of touch domes to mechanical stimulation by using drugs thought to interfere with calcium entry in other preparations.

Some preliminary results based on the isolated feline hind limb perfusion model and intra-dermal injection model suggest that Co^{2+} and verapamil block the response of SA1 mechanoreceptors to mechanical stimulation (Cooksey et al 1984). These results are verified in these models and the isolated rat skin-nerve preparation in the following chapter.

4.2 EXPERIMENTAL PROCEDURE

Two different models were used to test the effects of the inorganic ions Co^{2+}, Cd^{2+} and Mg^{2+} (as their chloride salts) and the phenylalkylamine verapamil hydrochloride:

1) The isolated feline hind limb perfusion model (Chap. 2.5) was used to test the effect of cadmium chloride (0.5, 1.0 and 2.5mM) and verapamil hydrochloride (100uM).

2) Superfusion of the isolated rat skin-nerve preparation with $CdCl_2$ (0.05, 0.1, 0.5 or 1.0mM), verapamil hydrochloride (20, 50 or 100uM) or $MgCl_2$ (5mM) (Chap 2.7).

Data analysis was carried out as described in

chapter 2.8. However, it was not possible to analyse the inter-spike interval distribution in these experiments because there were too few intervals in one response once the effect of the drug was established and insufficient stationarity between the responses to group several of them together.

b 4.3 RESULTS

In both experimental models the overall effect of the calcium channel blockers was to decrease the response to mechanical stimulation dose-dependently.

The feline isolated hind limb perfusion model

Either saline (0.15mM) or cadmium chloride was given in a 1ml bolus dose after the fifth mechanical stimulus. 15 minutes after the saline injection there was no change in the response (Fig. 4.1). The response to mechanical stimulation declined after the injection of $CdCl_2$ and a summary of results is given in Table 4.1. One minute after injection, 0.5mM $CdCl_2$ significantly increased the response of the touch domes to mechanical stimulation. With this exception , $CdCl_2$ caused a dose dependent decline in the response of the SA1 mechanoreceptors. Even after the response had failed it was still possible to elicit a response from the afferent nerve fibre when it was electrically stimulated.

One experiment was carried out in which 10ml of either saline (0.15mM) or 100uM verapamil hydrochloride were injected into the localised limb circulation of the

FIGURE 4.1

This graph illustrates the normalized response of touch domes to mechanical stimulation in the feline isolated hind limb perfusion model. Saline (1.0ml) was injected into the isolated circulation after the fifth mechanical stimulus (arrow). Stimuli were applied at 30s intervals with a stimulus duration of 5s. Saline neither attenuates nor enhances the mechanosensitivity of the touch domes.

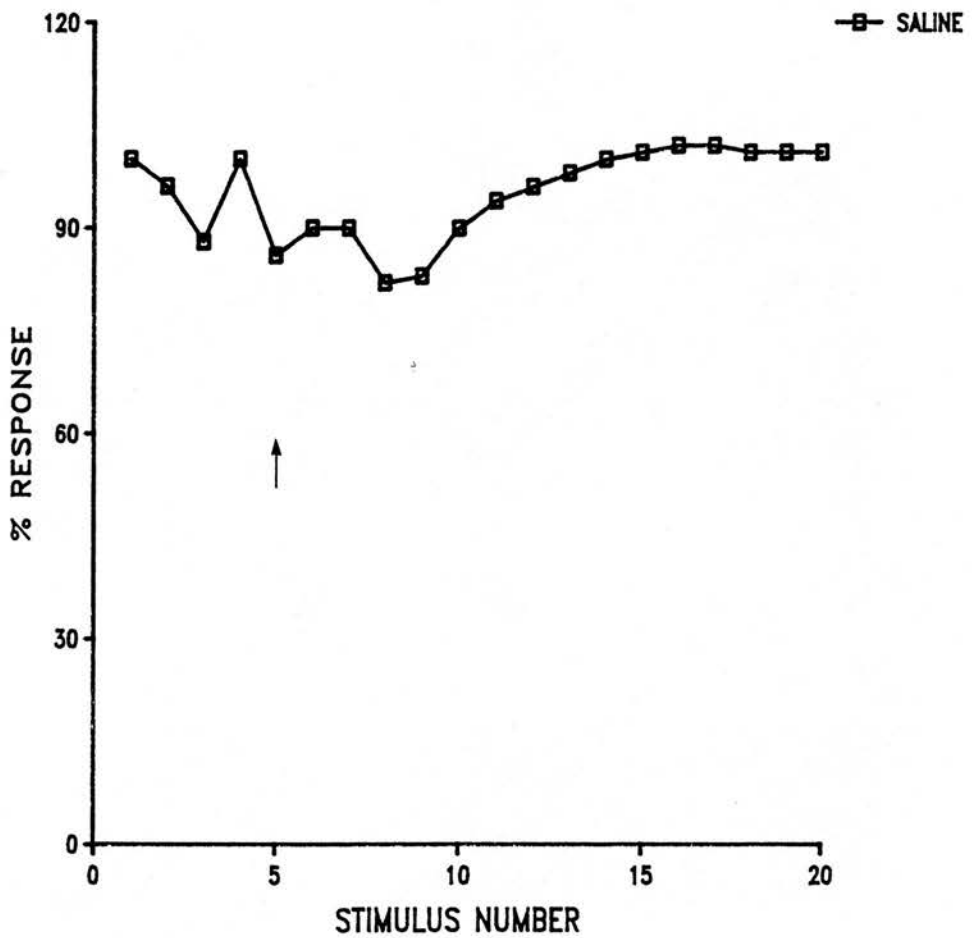

SALINE
120
90
60
30
0
% RESPONSE
0
5
10
15
20
STIMULUS NUMBER

TABLE 4.1

The effect of $CdCl_2$ on the response of SA1 mechanoreceptors in the feline isolated hind limb perfusion model.

TIME AFTER $CdCl_2$ (mins)	CONTROL (n=5) (30-56 cs^{-1})	0.5mM (n=2) 50, 53 cs^{-1}	1.0mM (n=2) 31, 41 cs^{-1}	2.5mM (n=2) 41, 41 cs^{-1}
1	100±7.7	130±8.7*	98±15.9	65±2.1*
2	93±8.9	115±3.0	88±8.3	13±7.0**
5	103±7.6	101±15.7	82±0.9*	0
8	98±8.8	95±0.6	79±3.8	0

(MEAN ± SE)

n= number of experiments; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Raw data given as counts per second (cs^{-1}).

cat. The results of this experiment are shown in Figure 4.2. 12 minutes after the injection of saline the response was 91% of the control value. After verapamil the response of the touch dome gradually declined to approximately 50-60% of the control value 9 minutes after the injection.

The isolated rat skin-nerve preparation

Five stimulations were given before the preparation was exposed to drugs to ensure that the touch domes were responding in the usual way. Cadmium chloride caused an attenuation in the response of the SA1 mechanoreceptors compared to the controls (Fig. 4.3). This effect was dose dependent, the higher concentrations causing the response to decline most quickly (Table 4.2). Fifteen minutes after application the responses were reduced to 60 $\pm$ 7.9, 50 $\pm$ 2.7, 32 $\pm$ 10.4 and 23 $\pm$ 5.6% by 0.05, 0.1, 0.5 and 1.0mM $CdCl_2$ respectively ($P < 0.01$ at 0.05mM and $P < 0.001$ at 0.1, 0.5 and 1.0mM). The effect of $CdCl_2$ on the dynamic and static phases of the response was also observed. 0.05mM $CdCl_2$ had no effect on the dynamic response while 0.1 and 0.5mM $CdCl_2$ attenuated the response but to a lesser degree than the maximum dose (Table 4.3). Only the highest dose of $CdCl_2$ administered (1.0mM) reduced the response to a value comparable to its effect on the whole response (Table 4.3). $CdCl_2$ caused a dose dependent attenuation in the static response of the touch domes which was very similar to its effects on the whole response (Table 4.4). The ID_{50} value for the dynamic

FIGURE 4.2

This graph illustrates the normalized responses of touch domes to mechanical stimulation in the single feline isolated hind limb perfusion model experiment after injection of saline or 100uM verapamil. Stimuli were applied once per minute with a stimulus duration of 5s. 5 stimuli were applied before the injection of either saline (10ml) or verapamil (10ml). Verapamil reduced the response to ~ 50% of its original level.

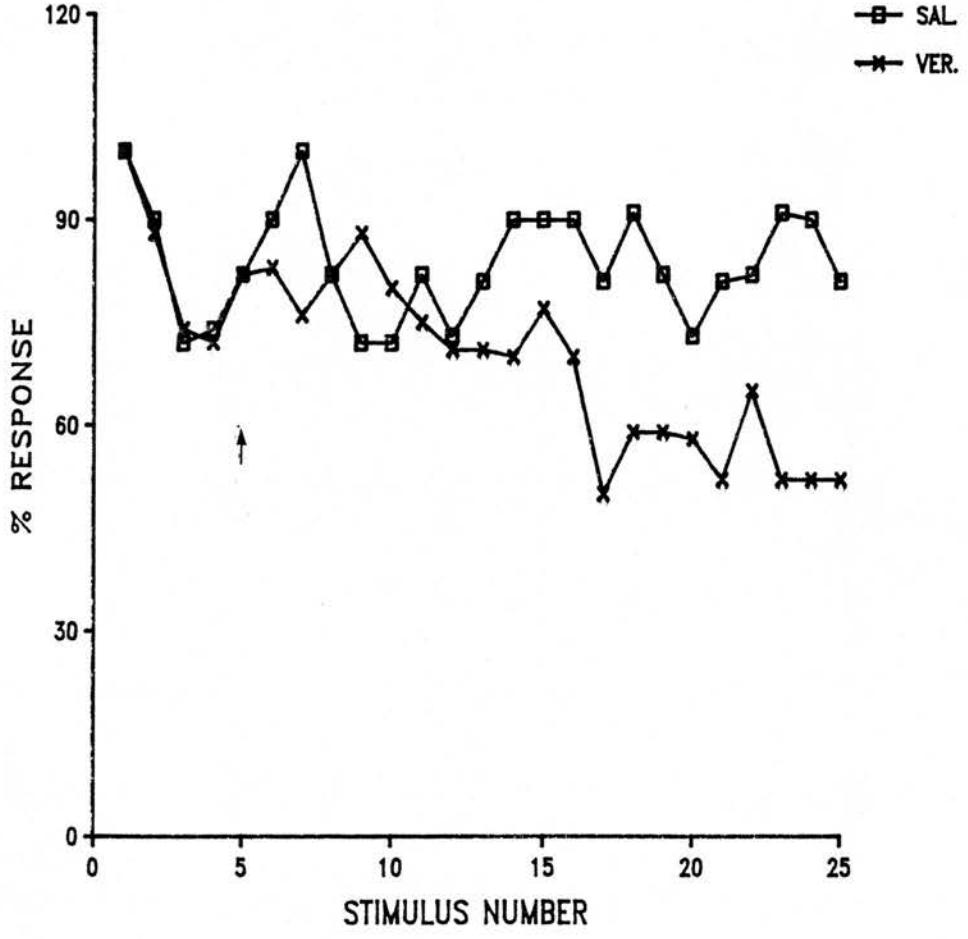
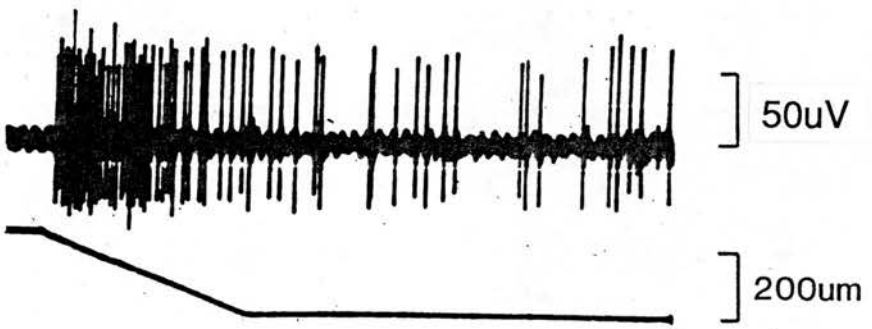

SAL.
VER.
120
90
60
30
0
% RESPONSE
0
5
10
15
20
25
STIMULUS NUMBER

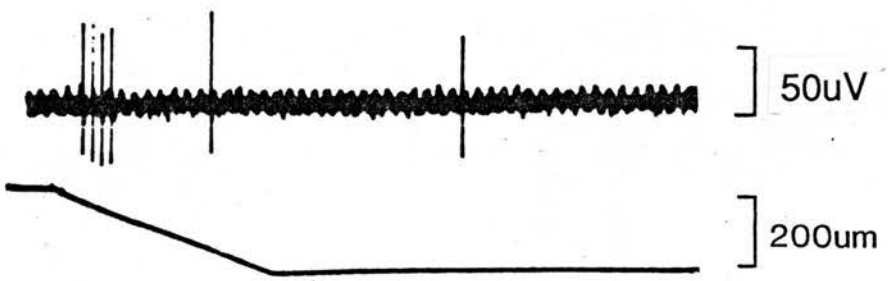
FIGURE 4.3

Illustrated are oscill oscope traces recorded in the isolated rat skin-nerve preparation before and after superfusion with $CdCl_2$. The top trace shows the control response obtained before $CdCl_2$. The bottom trace shows how the response declined after superfusion with $CdCl_2$ for 7 minutes. The mechanical stimulation is shown as the downward deflection in the lower half of each trace.

Control response



Response after 1.0mM cadmium chloride



0.1ms

TABLE 4.2

The effect of $CdCl_2$ on the whole response of SA1 mechanoreceptors in the isolated rat skin nerve preparation.

STIM. NO.	CONTROL (n=7) 22-37cs^{-1}	0.05mM (n=3) 17-51cs^{-1}	0.1mM (n=4) 23-57cs^{-1}	0.5mM (n=5) 26-34cs^{-1}	1.0mM (n=5) 25-64cs^{-1}
1	100	100	100	100	100
2	99±0.6	98±4.0	101±1.0	98±1.5	100±0.2
3	98±1.4	102±3.3	98±0.5	98±2.2	98±1.6
4	101±2.0	98±5.5	95±1.7	93±3.1	97±1.1
5	105±2.7	100±6.5	92±3.5	88±3.4	91±2.0
6	103±2.4	95±5.1	91±3.2	95±5.8	86±3.6*
7	98±2.3	87±4.0	86±2.9	81±5.5*	76±3.1**
8	105±1.2	89±6.4	82±2.2***	75±6.0***	71±2.3***
9	98±1.4	83±3.2**	83±2.0**	79±8.0	66±5.2***
10	96±1.7	78±4.3*	80±4.4*	72±11.0	60±5.4***
11	98±1.6	83±8.5	74±2.2***	62±10.2**	54±4.9***
12	99±2.8	73±8.4*	70±1.9**	59±9.4**	47±5.5***
13	95±1.3	77±9.3	71±2.3***	51±12.6**	46±5.6***
14	98±1.5	71±14.2	69±1.6***	49±11.7***	40±6.1***
15	98±2.3	71±11.8	67±3.6***	46±11.0***	38±5.3***
16	91±1.6	71±12.0	64±3.7***	43±11.0**	34±5.8***
17	91±1.5	68±13.6	63±5.4**	42±9.6***	30±6.0***
18	92±1.1	66±8.8*	55±4.4***	40±10.0***	28±6.0***
19	96±1.9	62±9.1*	51±3.3***	35±10.2***	25±5.8***
20	93±1.4	60±7.9**	50±2.7***	32±10.4***	23±5.6***

(mean ± SE).

n=number of experiments; * P < 0.05; **P < 0.01; *** P < 0.001

5s Stimuli were applied at 30s intervals.

Raw data given as counts per second (cs^{-1}).

TABLE 4.3

The effect of $CdCl_2$ on the dynamic response of SA1 mechanoreceptors in the isolated rat skin-nerve preparation.

STIM. NO.	CONTROL (n=6) 55-160 cs^{-1}	0.05mM (n=3) 50-140cs^{-1}	0.1mM (n=4) 90-145cs^{-1}	0.5mM (n=5) 55-100cs^{-1}	1.0mM (n=5) 60-181cs^{-1}
1	100	100	100	100	100
2	97±3.2	104±5.8	88±6.5	95±6.5	98±4.0
3	98±6.2	102±15.6	87±5.2	91±3.9	101±9.3
4	102±6.4	96±4.0	86±5.9	93±6.7	86±5.5
5	96±8.2	101±1.3	84±6.5	103±12.5	91±10.2
6	93±6.9	97±3.3	85±9.8	90±6.1	85±7.0
7	98±7.2	92±9.3	86±5.9	100±8.5	75±9.5
8	104±4.4	98±8.9	85±9.1	89±12.0	81±13.5
9	110±6.6	104±7.2	83±3.9*	87±7.8*	67±9.2**
10	106±9.4	104±5.8	84±5.8	83±10.1	59±11.2**
11	99±12.0	102±6.5	74±2.6	87±14.3	57±10.8*
12	103±8.8	94±7.3	77±3.6	88±11.5	55±7.2**
13	93±6.7	95±7.6	80±4.7	83±8.9	52±7.1**
14	93±6.7	95±2.9	76±4.8	80±9.0	54±9.8**
15	91±6.4	83±4.8	68±9.6	73±10.5	47±8.1**
16	85±7.4	92±2.0	71±6.8	68±8.8	37±7.9**
17	88±4.8	77±5.1	65±7.6*	63±10.0*	45±9.9**
18	91±4.7	83±4.0	61±6.7**	60±11.4*	36±14.7***
19	87±4.6	76±4.3	56±8.8*	61±9.9*	33±8.5***
20	91±6.0	80±2.3	50±8.2**	60±16.3	31±5.4***

(MEAN ± SE)

n= number of experiments; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

55 Stimuli were applied at 30s intervals.

Raw data given as counts per second (cs^{-1}).

TABLE 4.4

The effect of $CdCl_2$ on the static response of SA1 mechanoreceptors in the isolated rat skin-nerve preparation.

STIM. NO.	CONTROL (n=6) 19-34 cs^{-1}	0.05mM (n=3) 14-47 cs^{-1}	0.1mM (n=4) 15-47 cs^{-1}	0.5mM (n=5) 14-30 cs^{-1}	1.0mM (n=5) 21-50 cs^{-1}
1	100	100	100	100	100
2	98±1.6	100±8.6	99±2.3	102±3.0	95±4.0
3	105±8.2	101±5.6	96±3.2	96±2.5	98±2.9
4	108±9.5	98±3.0	94±4.9	92±5.6	96±3.4
5	102±11.4	98±4.2	93±4.5	98±7.1	87±5.4
6	105±7.6	95±8.4	87±1.1	84±7.3	76±6.1*
7	104±8.2	89±5.3	81±3.9	76±8.9	72±3.5**
8	104±5.1	85±6.9	82±4.7*	84±7.9	64±6.0***
9	102±4.3	84±7.5	79±7.1*	77±16.0	60±6.4***
10	98±4.6	76±9.2	75±3.6**	67±12.3*	55±5.4***
11	97±7.2	74±6.8	70±1.6*	60±11.5*	48±7.2**
12	90±4.4	75±7.4	70±6.2*	48±14.1*	48±7.3***
13	93±8.7	70±8.1	64±4.6*	54±11.8*	41±6.7**
14	96±5.9	66±9.0*	68±4.8*	50±12.3*	36±5.5***
15	90±4.8	55±11.1*	61±5.0**	51±11.4*	33±6.7***
16	95±5.0	64±14.4	65±6.3**	42±12.6**	30±6.7***
17	88±4.1	55±8.8*	53±5.6**	43±12.0*	27±6.1***
18	89±1.4	48±9.3**	48±3.8***	41±14.7*	23±5.9***
19	94±4.5	50±4.1***	50±4.5***	37±10.8**	22±5.7***
20	93±2.0	46±3.7***	52±5.7***	29±12.8**	22±5.3***

(MEAN ± SE)

n= number of experiments; * P < 0.05; ** P < 0.01; *** P < 0.001

5s Stimuli applied at 30s intervals.

Raw data given as counts per second (cs^{-1}).

response was larger than that for the static but not significantly so (0.36 + 0.3mM and 0.06 + 0.3mM respectively; $P > 0.05$, students t-test).

A comparison of the slopes of the graphs showing how the response declines between the 5th and the 20th stimuli is shown in Fig. 4.4. At each dose of $CdCl_2$ the slope was significantly lower than the control and this effect was dose dependent (- 0.74 $\pm$ 0.15, -2.35 $\pm$ 0.16, -2.76 $\pm$ 0.11 -4.11 $\pm$ 0.23 and -4.54 $\pm$ 0.21 for control, 0.05, 0.1, 0.5 and 1.0mM $CdCl_2$ respectively; mean $\pm$ s.e. $P < 0.001$, students t-test).

Verapamil hydrochloride caused a decline in the response of touch domes to mechanical stimulation compared to control values. This reduction in the response was dose dependent: 70 $\pm$ 1.7, 45 + _ 4.5 and 32 $\pm$ 17.7% in the presence of 20, 50 and 100uM verapamil respectively ($P < 0.001$, student's t-test), fifteen minutes after application (Table 4.5). Only the highest concentration of verapamil (100uM) had an attenuating effect on the dynamic component of the response, except 13 and 15 minutes after administration of 50uM verapamil (Table 4.6). Though the static component of the response was reduced to similar values as the whole response 15 minutes after application of verapamil, the time taken for the effect on the static response to become apparent was longer in the presence of 50 and 100uM verapamil and the effect was inconsistent at 20uM (Table 4.7). The ID_{50} values for the dynamic phase of the response was larger than that for the static but not

FIGURE 4.4

This graph illustrates the normalized responses of touch domes to mechanical stimulation in the isolated rat skin-nerve preparation after superfusion with $CdCl_2$ (0.05, 0.1, 0.5 or 1.0mM). Five stimuli were applied before the solution containing $CdCl_2$ was given (arrow). Stimuli were applied at 30s intervals with a duration of 5s. $CdCl_2$ caused a dose dependent decline in the response of the touch domes to mechanical stimulation.

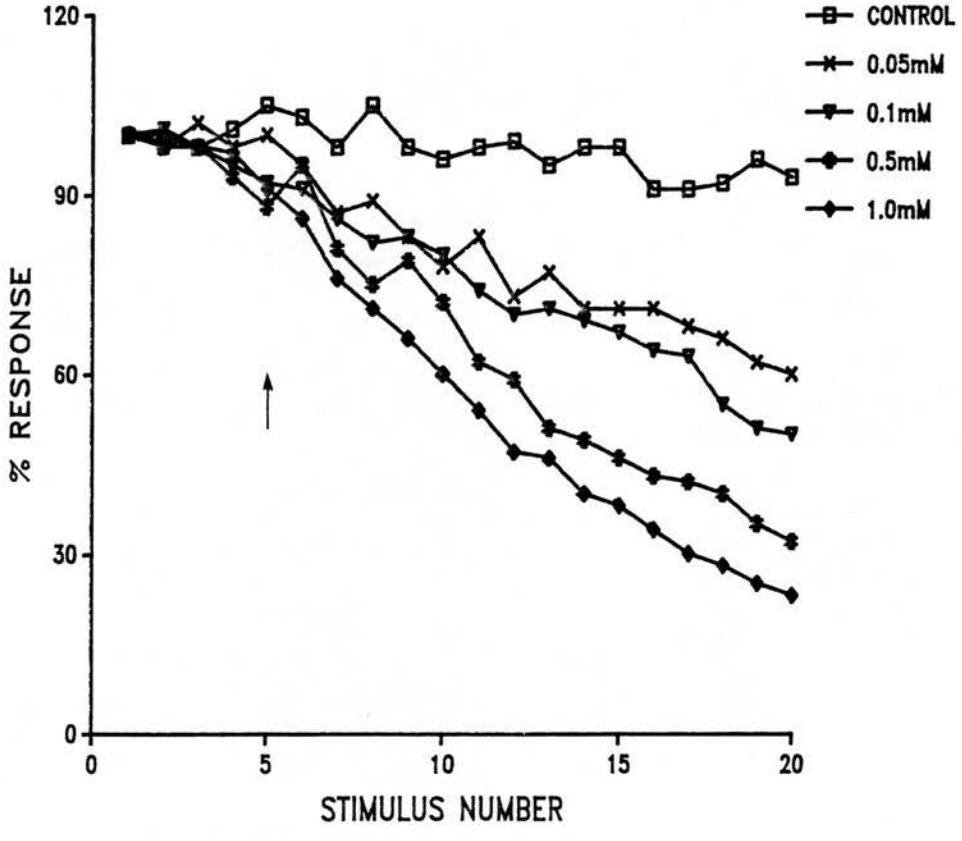

120
90
60
30
0
% RESPONSE
0
5
10
15
20
STIMULUS NUMBER
CONTROL
0.05mM
0.1mM
0.5mM
1.0mM

TABLE 4.5

The effect of verapamil hydrochloride on the whole response of SA1 mechanoreceptors in the isolated rat skin-nerve preparation.

STIM. NO.	CONTROL (n=7) 22-37cs^{-1}	20uM (n=2) 16, 32cs^{-1}	50uM (n=3) 21-30cs^{-1}	100uM (n=3) 27-30cs^{-1}
1	100	100	100	100
2	99±0.6	96±4.3	91±1.4	96±8.8
3	98±1.4	89±1.4	92±7.5	105±8.8
4	101±2.0	89±0.9	96±10.2	107±15.5
5	105±2.7	97±0.9	106±12.2	100±16.5
6	103±2.4	86±1.7*	106±3.2	92±12.3
7	98±2.3	83±8.5	112±10.7	86±20.5
8	105±1.2	68±6.8***	104±2.1	94±0.3**
9	98±1.4	65±11.3*	101±2.8	86±5.2
10	96±1.7	71±7.0*	93±10.2	76±3.3**
11	98±1.6	64±8.7**	98±11.0	67±8.0**
12	99±2.8	69±9.0*	88±8.4	59±7.1***
13	95±1.3	74±0.8***	81±2.9*	44±10.5**
14	98±1.5	68±1.4***	76±4.2**	38±7.2***
15	98±2.3	72±3.8**	63±6.5**	37±7.9***
16	91±1.6	71±3.0**	52±4.5***	32±10.3***
17	91±1.5	79±2.7***	62±0.0***	34±7.5***
18	92±1.1	77±7.4***	41±7.3***	33±10.9***
19	96±1.9	74±3.1***	44±2.8***	34±10.2***
20	93±1.4	70±1.7***	45±4.5***	32±10.7***

(MEAN ± SE)

n= number of experiments; * P < 0.05; ** P < 0.01; *** P < 0.001

5s Stimuli were applied at 30s intervals.

Raw data given as counts per second (cs^{-1}).

TABLE 4.6

The effect of verapamil hydrochloride on the dynamic response of SA1 mechanoreceptors in the isolated rat skin-nerve preparation.

STIM. NO.	CONTROL (n=6) 55-160cs^{-1}	20uM (n=2) 40,70cs^{-1}	50uM (n=3) 60-95cs^{-1}	100uM (n=3) 60-115cs^{-1}
1	100	100	100	100
2	97±3.2	93±7.0	85±5.8	96±6.2
3	98±6.2	87±1.0	105±15.9	95±6.7
4	102±6.4	94±6.0	83±4.2	90±6.1
5	96±8.2	80±5.5	102±16.2	78±8.1
6	93±6.9	87±1.0	106±3.2	87±13.0
7	98±7.2	94±6.0	105±7.0	87±13.0
8	104±4.4	81±19.0	104±4.0	71±16.5*
9	110±6.6	63±1.0**	94±6.7	52±11.8***
10	106±9.4	100±0.0	101±8.7	52±8.9**
11	99±12.0	75±25.0	114±11.3	56±5.4**
12	103±8.8	84±9.0	101±19.6	42±6.7**
13	93±6.7	104±3.5	81±9.7	38±4.5***
14	93±6.7	93±7.0	68±7.5	33±4.3***
15	91±6.4	80±5.5	75±17.0	37±6.0**
16	85±7.4	100±0.0	90±10.5	36±0.5**
17	88±4.8	87±1.0	68±0.5	38±9.5**
18	91±4.7	87±1.0	60±8.5*	43±0.3***
19	87±4.6	94±17.5	76±14.5	37±3.0***
20	91±6.0	96±3.5	51±4.8**	34±7.8**

(MEAN ± SE)

n= number of experiments; * P < 0.05; ** P < 0.01; *** P < 0.001

5s Stimuli were applied at 30s intervals.

Raw data given as counts per second (cs^{-1}).

TABLE 4.7

The effect of verapamil hydrochloride on the static response of SA1 mechanoreceptors in the isolated rat skin-nerve preparation.

STIM. NO.	CONTROL (n=6) 19-34cs^{-1}	20uM (n=2) 13,28cs^{-1}	50uM (n=3) 12-22cs^{-1}	100uM (n=3) 21-24cs^{-1}
1	100	100	100	100
2	98±1.6	95±5.0	94±4.4	104±13.0
3	105±8.2	86±1.5	95±4.8	109±14.7
4	108±9.5	80±3.5	95±12.2	116±21.7
5	102±11.4	84±4.5	106±8.3	110±22.5
6	105±7.6	76±2.0	104±4.0	91±23.8
7	104±8.2	71±8.0	108±12.0	93±23.9
8	104±5.1	68±4.5**	93±22.0	77±24.2
9	102±4.3	64±12.5**	102±7.6	76±22.0
10	98±4.6	72±3.5*	106±1.5	71±16.2
11	97±7.2	61±5.0*	96±2.5	62±19.9
12	90±4.4	72±6.5	92±1.8	56±13.8*
13	93±8.7	74±0.5	98±9.2	47±13.0*
14	96±5.9	67±6.0	92±12.2	41±9.2**
15	90±4.8	71±3.0	85±6.0	41±9.2**
16	95±5.0	72±5.0*	86±5.5	44±17.5*
17	88±4.1	82±0.5	81±33.0	35±5.0***
18	89±1.4	84±14.0	58±3.0***	34±9.5**
19	94±4.5	70±3.5*	63±10.2*	33±13.3**
20	93±2.0	68±0.0**	36±14.5**	32±12.7**

(MEAN ± SE)

n= number of experiments; * P < 0.05; ** P < 0.01; *** P < 0.001

55 Stimuli were applied at 30s intervals.

Raw data given as counts per second (cs^{-1}).

significantly so (61 ± 7.6 and 38 ± 9.4uM respectively; P > 0.05, student,s t-test),possibly because of the highly variable effects .

Linear regression analysis was used to calculate the slope of the graph showing how the response declined between the 5th and the 20th stimulations (Fig. 4.5). With the exception of 20uM verapamil, the slopes were significantly lower than that of the control and this effect was dose dependent (-0.74 ± 0.15, -5.07 ± 0.38 and -5.25 ± 0.45 for control, 50 and 100uM verapamil respectively; P < 0.001 student,s t-test).

$MgCl_2$ caused an attenuation in the response of SA1 mechanoreceptors to mechanical stimulation (Table 4.8). 15 minutes after the application the response was reduced to 48 ± 8.6 % (P < 0.01). 5mM $MgCl_2$ had no effect on the dynamic component of the response (Table 4.9). Table 4.10 shows that though 5mM $MgCl_2$ caused a decline in the static response this effect was less than that on the whole response. This is probably related to the higher standard errors associated with the mean values of response at each stimulation.

FIGURE 4.5

This graph illustrates the normalized responses of SA1 mechanoreceptors to mechanical stimulation in the isolated rat skin-nerve preparation after superfusion with a solution containing verapamil hydrochloride (20, 50 or 100uM). Five stimuli were applied before the drug solution was given. Stimuli were applied at 30s intervals and the stimulus duration was 5s. Verapamil caused a dose dependent decline in the response to mechanical stimulation.

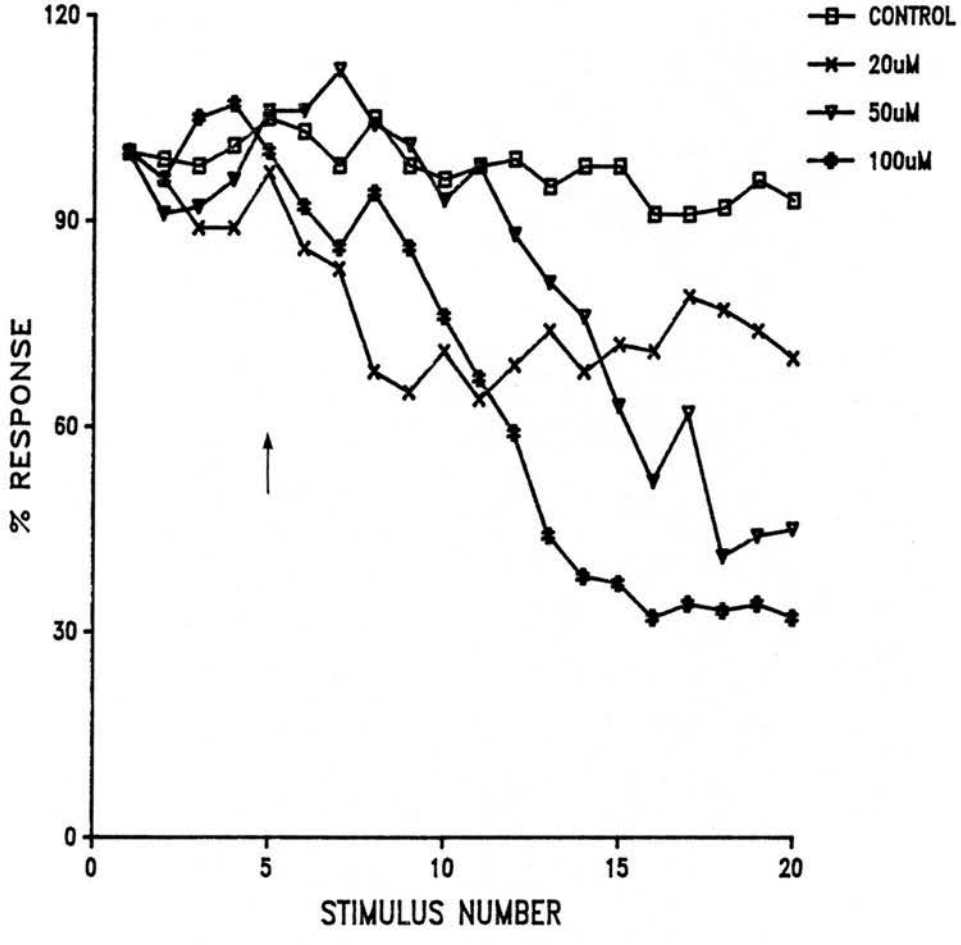

120
90
60
30
0
% RESPONSE
0
5
10
15
20
STIMULUS NUMBER
CONTROL
20uM
50uM
100uM

TABLE 4.8

The effect of $MgCl_2$ on the whole response of SA1 mechanoreceptors in the isolated rat skin-nerve preparation.

NO.	CONTROL (n=7) 22 - 37 cs^{-1}	5mM (n=5) 21 - 36 cs^{-1}
1	100	100
2	99±0.6	102±4.3
3	98±1.4	99±4.4
4	101±2.0	96±4.4
5	105±2.7	95±3.3
6	103±2.4	85±3.9*
7	98±2.3	79±6.0*
8	105±1.2	75±4.5***
9	98±1.4	76±6.2**
10	96±1.7	70±7.6*
11	98±1.6	69±7.3**
12	99±2.8	61±5.8**
13	95±1.3	60±7.6**
14	98±1.5	58±9.3**
15	98±2.3	55±8.2**
16	91±1.6	54±9.8**
17	91±1.5	51±8.4**
18	92±1.1	52±9.2**
19	96±1.9	49±8.8**
20	93±1.4	48±8.6**

(MEAN ± SE)

n= number of experiments; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$
5s stimuli were applied at 30s intervals.
Raw data given as counts per second (cs^{-1}).

TABLE 4.9

The effect of $MgCl_2$ on the dynamic response of SA1 mechanoreceptors in the isolated rat skin-nerve preparation.

STIM. NO.	CONTROL (n=6) 55 - 160 cs^{-1}	5mM (n=5) 40 - 120 cs^{-1}
1	100	100
2	97±3.2	105±8.3
3	98±6.2	99±7.8
4	102±6.4	104±7.0
5	96±8.2	92±6.4
6	93±6.9	92±8.1
7	98±7.2	87±5.6
8	104±4.4	89±7.5
9	110±6.6	87±10.2
10	106±9.4	89±9.4
11	99±12.0	91±15.8
12	103±8.8	79±8.5
13	93±6.6	78±7.7
14	93±6.7	84±11.2
15	91±6.4	75±11.2
16	85±7.4	75±8.5
17	88±4.8	80±10.1
18	91±4.7	81±13.1
19	87±4.6	69±12.1
20	91±6.0	66±15.5

(MEAN ± SE)

n= number of experiments; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

5s stimuli were applied at 30s intervals.
Raw data given as counts per second (cs^{-1}).

TABLE 4.10

The effect of $MgCl_2$ on the static response of SA1 mechanoreceptors in the isolated rat skin-nerve preparation.

STIM. NO.	CONTROL (n=6) 19 - 34 cs^{-1}	5mM (n=5) 18 - 31 cs^{-1}
1	100	100
2	98±1.6	110±3.5
3	105±8.2	104±3.7
4	108±9.5	104±3.8
5	102±11.4	103±4.0
6	105±7.6	86±5.8
7	104±8.2	81±9.4
8	104±5.1	78±6.2**
9	102±4.3	81±7.9*
10	98±4.6	69±9.7*
11	97±7.2	74±10.9
12	90±4.4	66±4.2
13	93±8.7	64±13.0
14	96±5.9	61±13.8
15	90±4.8	57±13.3*
16	95±5.0	68±15.2
17	88±4.1	55±14.6
18	89±1.4	53±14.8
19	94±4.5	54±12.7*
20	93±2.0	53±13.4*

(MEAN ± SE)

n= number of experiments; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

5s stimuli were applied at 30s intervals.
Raw data given as counts per second (cs^{-1}).

A comparison of the slopes of the graphs showing how the response declines between the 5th and 20th stimuli showed that in the presence of $MgCl_2$ the slope was significantly lower than the control (Fig. 4.6) ; -0.74 $\pm$ 0.16 and -2.87 $\pm$ 0.20 (mean $\pm$ s.e.) for control and 5mM $MgCl_2$ ($P < 0.001$, student's t-test).

The effect of $CdCl_2$, $MgCl_2$ and verapamil hydrochloride on the response of SA1 mechanoreceptors to mechanical stimulation were generally the same. Table 4.11 shows the rank order of potency of these drugs as inhibitors of activity in the afferent fibres, based on the ID_{50} values calculated for the whole response. This table shows that verapamil hydrochloride is the most potent and, therefore the most effective at inhibiting the response, and magnesium chloride the least effective.

4.4 DISCUSSION

All of the calcium entry blockers had the same general effect of decreasing the response of the touch domes to mechanical stimulation, irrespective of which preparation they were tested in.

Several inorganic cations including Cd^{2+}, Co^{2+} and Mg^{2+}

FIGURE 4.6

This graph illustrates the response of touch domes to mechanical stimulation in the isolated rat skin-nerve preparation after superfusion with a solution containing $MgCl_2$ (5mM). Stimuli were applied at 30 s intervals and the stimulus duration was 5s. Five mechanical stimulations were given before the drug solution was superfused. $MgCl_2$ caused a decline in the response to mechanical stimulation.

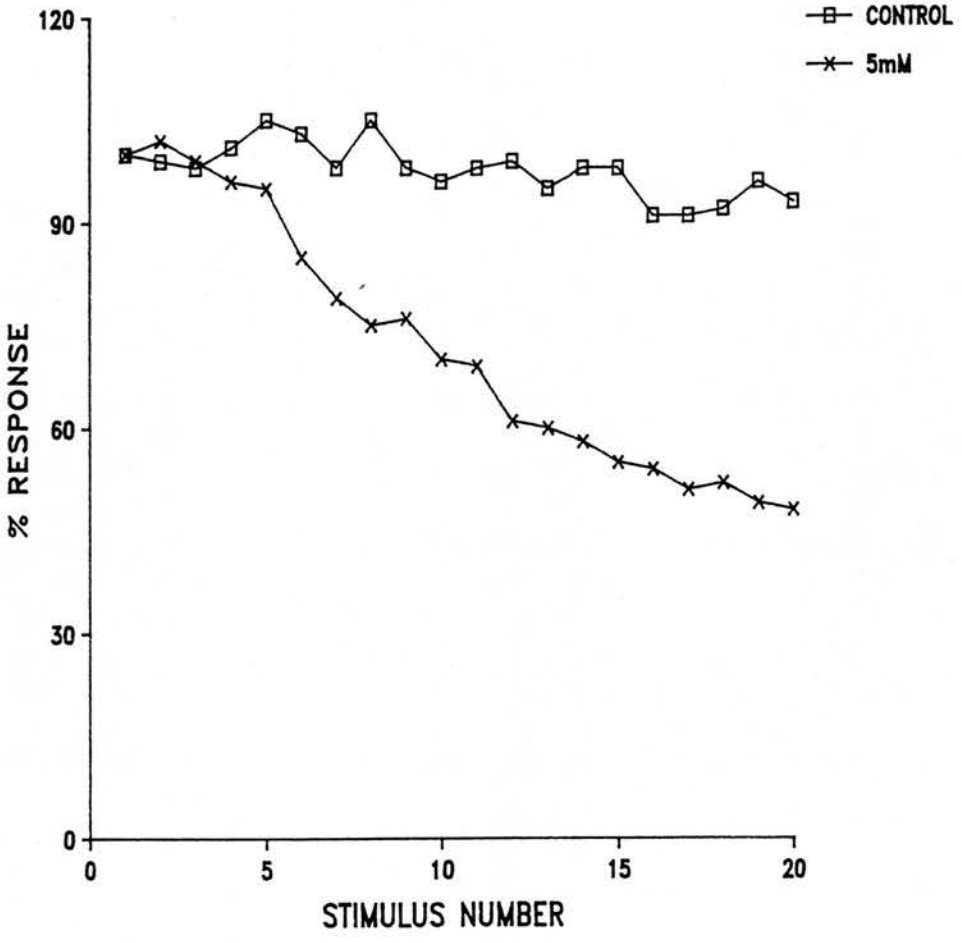

CONTROL
5mM
120
90
60
30
0
% RESPONSE
0
5
10
15
20
STIMULUS NUMBER

TABLE 4.11

Rank order of potency of verapamil hydrochloride, $CdCl_2$ and $MgCl_2$ as inhibitors of SA1 mechanoreceptors.

DRUG	DOSE RANGE (uM)	ID_{50} (uM)	NUMBER OF EXPERIMENTS
VERAPAMIL HYDROCHLORIDE	20 - 100	45±3	8
CADMIUM CHLORIDE	50 - 1000	110±10	17
MAGNESIUM CHLORIDE	5000 - 15000	4810±1120	15

(MEAN ± SE)

can interact with calcium channels in a direct and reversible manner (Hille, 1984). The type of influence depends on two factors: a) the affinity of the cation for the channel binding site and b) the speed with which the ion traverses the aqueous pore. Cd^{2+}, Co^{2+} and Mg^{2+}have high affinities for the channel binding site but traverse the pore at low, even negligible speeds. Hence the cations behave as potent competitive inhibitors of ion permeation through calcium channels (Hille, 1984).

$CoCl_2$ and $CdCl_2$ caused a dose dependent attenuation in the response of the touch domes mechanically stimulated in the <u>in vivo</u> cat models and $CdCl_2$ and $MgCl_2$ caused a dose dependent decline in the response of SA1 mechanoreceptors in the <u>in vitro</u> preparation. Increasing concentrations of magnesium produced a progressive decrease in the end plate potential at the frog neuromuscular junction (NMJ) (del Castillo and Stark 1952; del Castillo and Engbaek 1954). Cobalt has a similar effect at the frog NMJ (Weakly 1973). It also produced a dose dependent decline in the epp which almost disappeared at 1mM Co^{2+}. It is generally accepted that these cations inhibit neuromuscular transmission by competitive antagonism of Ca^{2+}, leading to failure of acetylcholine release.

Cobalt (3mM) also blocked the K^+-evoked release of substance P from cultured rat dorsal root ganglion neurones, [^{3}H]-noradrenaline from cultured rat sympathetic neurones (Preney et al 1986) and dopamine from isolated

tuberoinfundibular neurones (Annunziato, Amoroso, Tagliatela, De Natale and Di Renzo 1986).

It is believed that a transient rise in the concentration of intracellular Ca^{2+} is the trigger for stimulus-secretion coupling. A transient increase in ionized intracellular calcium concentration occurs when isolated bovine adrenal medullary cells are challenged with either acetylcholine or high K^{+}; there is no increase in intracellular Ca^{2+} in the absence of extracellular Ca^{2+}. Mg^{2+} (5mM) blocks the increase in intracellular Ca^{2+} and the secretory response of the isolated medullary cells (Knight and Kesteven 1983).

The similarities of the action of Mg^{2+}, Co^{2+} and Cd^{2+} on SA1 mechanoreceptors and the NMJ and the demonstration that they can block evoked transmitter release in vitro suggests they may be preventing entry of extracellular calcium and therefore the transient increase in intracellular Ca^{2+}, hence blocking transmitter release from Merkel cell-neurite complexes.

Verapamil reduced the response of SA1 mechanoreceptors when tested in the isolated hind limb perfusion model.

It also caused a dose dependent attenuation in the response of the touch domes when tested in the isolated preparation. Verapamil and D600 blocked the K^{+} and acetylcholine evoked release of catecholamines from perfused adrenal glands in situ at ID_{50}'s of at least 10uM

(Aqueros and Daniels 1978; Pinto and Trifaro 1976). D600 (50 and 100uM) inhibited the K^+-evoked release of dopamine from tuberoinfundibular neurones in vitro (Annunziato et al 1986) and D600 (0.5mM) blocked the intracellular calcium transient and secretory response in isolated bovine medullary cells (Knight and Kesteven 1983). In the present study verapamil blocked the response of rat touch domes to mechanical stimulation at a similar concentration (ID_{50} 45 $\pm$ 3uM) suggesting that it is inhibiting transmitter release. How does verapamil block the response of SA1 mechanoreceptors to mechanical stimulation? Phenylalkylamines are probably sufficiently lipophilic to penetrate the cell membrane and enter the cytosol. D600 shortened and lowered the calcium plateau of the action potential in guinea-pig ventricular myocotes whether it was applied extra- or intracellularly. However, its polarized N-methyl quaternerized derivative only showed this action when applied intracellularly (Heschler, Pelzer Trube and Trautwein 1982). This suggests that the phenylalkylamines exert their calcium blocking effects intracellularly.

Although the inhibitory effect of verapamil and D600 can be reversed by increasing the extracellular calcium concentration, it is unlikely that they behave in a simple competitively antagonistic manner like the inorganic ions. This conclusion is based on experiments which show that cadmium blocks barium currents more strongly than calcium currents (which would be expected since barium has the higher affinity for the calcium binding site) whereas D600

has the opposite effect (Lee and Tsien 1983).

Recently three subtypes of calcium channel have been demonstrated in chick dorsal root ganglion cells, namely L, T and N (Nowycky, Fox and Tsien 1985). They are classified on the basis of the size of their conductance. L channels have a large conductance which contributes a long lasting current at strong depolarizations; T-channels have a relatively tiny conductance which underlies a transient current activated at weak depolarizations; N-channels have an intermediate conductance and require strong depolarizations for activation and strong negative potentials for complete removal of inactivation. The channel subtypes also show differential sensitivity to various calcium channel modulators. The dihydropyridine agonist Bay K8644 (5uM) increased the probability of opening of L-type channels in isolated chick dorsal root ganglion neurones (Nowycky et al 1985) and cultured guinea-pig ventricular myocytes (Nilius, Hess, Lansman and Tsien 1985). In the latter preparation nimodipine (5uM) halved the average L-type channel current but did not change the T-type current (Nilius et al 1985). The evoked release of neurotransmitters from neurones has generally been dihydropyridine insensitive (Miller and Freedman 1984) which may suggest that the L-type channel is not involved in neurotransmitter release, but there are exceptions (Perney, Hirning, Leeman and Miller 1986). Cadmium also has differential effects on the calcium channel subtypes. Cadmium (50uM) blocked L- and N-type

channels and reduced the current carrying capacity of the T-type channels to ~55% in chick dorsal root ganglion neurones (Nowycky, Fox and Tsien 1984) and 10uM cadmium reduced L-type but not T-type activity in guinea-pig ventricular myocytes (Nilius et al 1985). In the isolated rat skin-nerve preparation $CdCl_2$ blocked the response of SA1 mechanoreceptors with an ID_{50} of 110uM. There is, however, insufficient evidence in the present results to classify the calcium channel subtype which may be involved in SA1 mechanoreceptors. One way to investigate this would be to patch clamp isolated Merkel cells and determine the biophysical properties and drug sensitivity of the calcium channels present.

Analysis of the dynamic and static components of the response in the isolated preparation showed that the static component of the response was more sensitive than the dynamic component to all calcium blockers tested. A similar result was obtained when slowly adapting mechanoreceptors in the non-warty skin of frog were exposed to manganese (0.1 - 10mM) and magnesium (1 - 20mM) in the perfused frog leg preparation (Yamashita, Ogawa and Taniguchi 1986). Ft 1 mechanoreceptors, which fire irregularly like mammalian SA1 mechanoreceptors, showed a dose dependent reversible decline in response to mechanical stimulation; the dynamic and static responses were reduced to 50% by 10mM and 0.4mM manganese and and 20 and 14mM magnesium respectively. The ID_{50} values for $MgCl_2$ in the isolated rat skin-nerve preparation were 8.6 $\pm$ 0.5 and 6.5 $\pm$ 2.0mM respectively. Hence the dynamic component

of the response appears to be more resistant to calcium blockers than the static component.

These results are discussed further in chapter 5.

CHAPTER 5

DISCUSSION and CONCLUSIONS.

DISCUSSION and CONCLUSIONS

The aim of the work in this thesis was to investigate the hypothesis that chemosynaptic transmission may be involved in the transduction process of cutaneous SA1 mechanoreceptors. Immunohistochemical studies suggested a potential chemical transmitter in rodents was a met-enkephalin-like substance (Hartschuh et al 1979).

The results presented in chapter 3 suggest that the met-enkephalin-like substance is not the excitatory transmitter in cutaneous SA1 mechanoreceptors in the rat but that it does play a modulatory role by attenuating the response to mechanical stimulation. Definition of the precise physiological role of the met-enkephalin-like substance requires determination of both the location and the classification of the opioid receptor involved. The two predominant actions of opioids on the nervous system are the depression of neuronal excitability and the inhibition of neurotransmitter release. Unlike many other sensory receptors, SA1 mechanoreceptors do not spontaneously discharge. The results with naloxone in the *in vivo* rat preparation suggest that an opioid substance is released tonically as has been demonstrated in the cat carotid body (Monti-Bloch and Eyzaguirre, 1985). Perhaps met-enkephalin is involved in preventing spontaneous activity by preventing neurotransmitter release or by reducing nerve terminal sensitivity.

The cellular mechanisms underlying the effects of opioids have been investigated in several tissues

including the guinea-pig myenteric plexus, spinal cord and dorsal root ganglia preparations neuroblastoma X glioma hybrid cells and rat locus coeruleus neurones. Opioids have been shown to depress neuronal firing in rat locus coeruleus neurones by increasing potassium conductance, resulting in membrane hyperpolarization (Williams, Egan and North, 1982). Inhibition of neurotransmitter release may result from actions prior to or at the release site. It has been suggested that membrane hyperpolarization of pre-synaptic fibres in the guinea-pig myenteric plexus inhibited acetylcholine release (Cherubini, Morita and North, 1985). A decrease in the entry of calcium would also block neurotransmitter release. B-endorphin inhibits the uptake of $^{45}Ca^{++}$ in mouse brain both *in vivo* and *in vitro* (Guerrero-Munoz, Guerrero, Leongway and Hao Li, 1979). Opiates also indirectly modulate calcium entry into central neurones by shortening the duration of the action potential as a result of increased potassium conductance (North and Williams, 1983). It has been suggested that K-opiates directly depress calcium entry through voltage sensitive channels in the guinea-pig myenteric plexus (Cherubini and North, 1985) and cultured mouse dorsal root ganglion neurones (Werz and MacDonald, 1985). It is not possible to distinguish between these mechanisms in cutaneous SA1 mechanoreceptors.

The results presented in chapter 4 suggest that calcium is necessary for the normal response of touch domes to mechanical stimulation. Calcium entry is required for stimulus-secretion coupling (Baker and Knight, 1978).

It is possible that the inhibitory action of the calcium channel blockers used in these experiments is due to failure of neurotransmitter release from the cutaneous SA1 mechanoreceptors. In view of the cellular mechanisms underlying the actions of opioids discussed above, both met-enkephalin and the calcium channel blockers may be acting by the same mechanism. It should be possible to test this idea by superfusing the isolated rat skin-nerve preparation with a solution containing both types of drug. If they are acting by the same mechanism then their sum effect should be no greater than either of them acting alone.

The results found with the calcium channel blockers are in general agreement with those found in frog slowly adapting cutaneous mechanoreceptors (Yamashita, Ogawa and Taniguchi, 1986). The effect of calcium channel blockers has also been investigated in other sensory receptors. During ramp and hold stretch of the frog muscle spindle the dynamic response was decreased by calcium channel blockers (Ito, Komatsu and Katsuta, 1981). The calcium spikes reported in terminals of the frog muscle spindle occur at the completion of the ramp stretch and this corresponds to the phase where the calcium channel blockers exert their greatest influence. The effect of changing calcium levels on the activity of cold receptors has also been tested (Schafer, Braun and Hensel, 1982). At constant temperatures increased calcium concentrations reduced the discharge rate and decreased calcium levels

increased the discharge rate of firing elicited in previously non-firing fibres. During cooling steps the peak dynamic response was not generally affected. These results seem to be in conflict with those described above. However, the authors explain the results by assuming that removal of extracellular calcium reduced a calcium-dependent hyperpolarizing outward current and therefore depolarized the membrane while increased extracellular calcium enhanced the hyperpolarizing current.

The effect of varying calcium levels on the responses of cutaneous mechanoreceptors in an isolated salamander skin preparation has also been reported (Diamond, Holmes and Nurse, 1986). Removal of extracellular calcium with increased magnesium levels had no significant effect the sensitivity of the mechanoreceptors. Cobalt (2-10mM) decreased or abolished the responses of the mechanoreceptors to mechanical stimulation. This effect persisted even when the epidermis and therefore the Merkel cells were removed. The authors argued that these findings were evidence against the involvement of chemosynaptic transmission in the transduction process. It has been reported that Merkel cells in salamander and Xenopus skin form rapidly adapting mechanoreceptors (Parducz, Leslie, Turner and Diamond 1977; Mearow and Diamond 1988) whereas other workers have reported that they form slowly adating mechanosensitive receptors in Rana skin (Ogawa, Morimoto and Yamashita 1981). This discrepancy may be explained by the fact that the plateau phase of the mechanical stimulus applied by Parducz et al (1977) and Mearow and Diamond

(1988) was only 0.2ms. It is possible that they were eliciting only the dynamic phase of the mechanosensory response. If this is the case, it may explain why the results of Diamond et al (1986) do not support the concept of chemosynaptic transmission. If this rapidly adapting response corresponds to the dynamic phase observed in rodent Merkel cells, then these results are in general agreement with the results presented in this thesis since the dynamic phase of the response was relatively insensitive to drugs (see below).

A consistent finding of the experiments on the SA1 mechanoreceptors was that the dynamic component of the response was less sensitive to all the drugs tested than the static component . This may be explained by different mechanisms underlying the two components of the response: the dynamic component could arise from mechanical deformation of the nerve ending whereas release of a transmitter substance may be responsible for the static component which corresponds to the slowly adapting phase of the response. This explanation is consistent with the findings that SA1 mechanoreceptors in sinus hair follicles of cats can follow stimulation frequencies of up to 1500Hz in a 1:1 fashion (Gottschaldt and Vahle-Hinz, 1981) and that nerve fibres regenerating to SA1 mechanoreceptors respond to mechanical stimulation non-specifically before they re-innervate the touch domes (Brown and Iggo, 1963). It has been reported that even after removal of Merkel cells from rat SA1 mechanoreceptors by skin irradiation,

it was possible to elicit a response to mechanical stimulation from the remaining nerve fibres (Diamond, Mills and Mearow,1988). Perhaps these responses were similar to those non specific responses described by Brown and Iggo (1963).

In the experiments described in this thesis it was not possible to distinguish whether the drugs were exerting their effects exclusively on the Merkel cells within the SA1 mechanoreceptors, though some attempts were made to do so. During some *in vivo* experiments the nerve fibres were electrically stimulated and appeared to function normally. In the isolated rat skin-nerve preparation the skin was stimulated manually after the responses of the SA1 mechanoreceptors had obviously decreased and it was possible to elicit responses from other types of cutaneous mechanoreceptor. However, quantitative studies are obviously required in this preparation. The most suitable mechanoreceptor to carry out a study on would be the SA11 mechanoreceptor.

An alternative way to test whether the drugs were acting only on the Merkel cells would be to dissociate the Merkel cell-neurite complex and investigate their electrophysiology separately. Recently Merkel cells were isolated from neonatal rat vibrissae and patch-clamped (Cooper and Nurse, 1986). The cell membrane was stretched by pipette suction and surface jets of extracellular fluid but neither of these procedures altered the membrane potential or cell gating. The authors suggested this

result indicated that the Merkel cell was not mechanosensitive. However, the membrane potential recorded from these cells was low (-30mV) which may be an indication that the cells were damaged. Alternatively, the Merkel cells may have been immature.

Some of the problems formerly associated with the use of pharmacologically active agents to investigate the function of SA1 mechanoreceptors in in vivo preparations have been overcome with the development of this new in vitro rat skin-nerve preparation. The preparation also had other advantages. There is usually a decline in the response of touch domes to constant displacement mechanical stimulation due to skin creep away from the mechanical stimulator. This was less of a problem in the in vitro preparation because the skin flap had a rigid support. Problems of this nature could be reduced even further if constant force mechanical stimulation was used in conjunction with the isolated rat skin-nerve preparation.

If the met-enkephalin-like substance found in rodent Merkel cells is not the transmitter substance, then what is ? Extracellular recordings from frog vestibular hair cell afferents suggest that glutamate, aspartate and other related acidic amino acids increased the firing rates of these afferents in a dose dependent manner (Annoni, Cochran and Precht, 1984). When synaptic transmission was blocked presynaptically using a high Mg^{++} / low Ca^{++} solution, glutamate, aspartate and quisqualic acid

reversibly depolarized the afferent fibres, suggesting a postsynaptic site of action for these substances. Acidic amino acid antagonists such as kynurenic acid (KENYA) and 2-amino-5-phosphovaleric acid (APV) reversibly reduced the amplitudes of the spontaneously firing of the afferent fibres but did not affect their frequency which suggests subsynaptic blockade. KENYA and APV also block the depolarizations induced by glutamate and aspartate. These results suggest that glutamate or other related acidic amino acids is the transmitter substance at this synapse. Given the morphological similarities between Merkel cells and hair cells it is possible that an acidic amino acid is the excitatory transmitter in SA1 mechanoreceptors. This hypothesis could be tested by superfusing the isolated rat skin-nerve with antagonists such as KENYA and APV. If this resulted in an attenuation of the response of the touch domes to mechanical stimulation, then selective agonists could be used to try and excite the SA1 mechanoreceptors in the absence of any mechanical stimulation.

A hypothetical model adapted from that of Iggo and Findlater (1984) for the function of individual Merkel cell-neurite complexes is represented schematically in diagram 5.1. A mechanical stimulus applied to the skin bends the cytoplasmic processes on the superficial side of the Merkel cell-neurite complex. This would activate mechanically gated ionic channels either in the membrane on the epidermal surface of the Merkel cell or on the cytoplasmic processes themselves ; extracellular calcium

FIGURE 5.1

Illustrated is a diagram of a Merkel cell-neurite complex showing the various steps in the proposed transduction process.

1) Mechanical stimuli applied to the skin cause deformation of the cytoplasmic processes (P) which 2) opens mechanically gated channels either at the base of the cytoplasmic processes or on the cytoplasmic processes themselves.

3) This allows Ca^{2+} to enter the Merkel cell causing 4) stimulus-secretion coupling and movement of the dense cored vesicles (G) towards the specialized junctions between the Merkel cell and the neurite (NP) where they fuse releasing the transmitter substance 5) (possibly glutamate ?).

6) This binds with receptors on the nerve terminal resulting in its depolarization 7). If sufficient transmitter is released an action potential is initiated in the afferent fibre (A) 8.

The calcium channel blockers interfere with Ca^{2+} entry (step 3). Met-enkephalin may block at several sites: presynaptically to hyperpolarize the Merkel cell membrane (step 5) ; postsynaptically to hyperpolarize the nerve ending (step 7); it could block Ca^{2+} entry and therefore inhibit stimulus-secretion coupling (step 3).

(N = Merkel cell nucleus; BM = basement membrane of the epidermis).

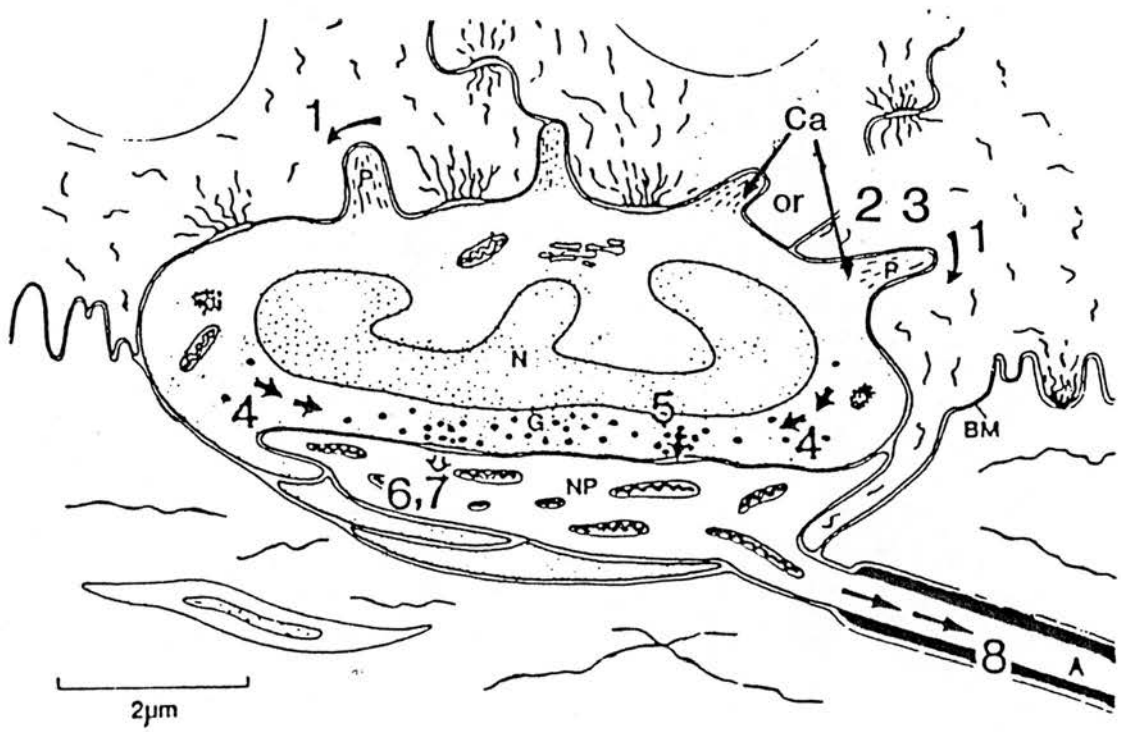


Fig. 5.1 Merkel cell-neurite complex showing the suggested sequence of events in the proposed transduction process

could now enter the cell. This would result in a transient rise of free intracellular calcium which would trigger stimulus secretion coupling. The dense cored vesicles containing the transmitter substance, possibly glutamate, would fuse with the Merkel cell membrane at the specialized synapse-like structures, releasing the transmitter substance into the synaptic cleft. The nerve terminal membrane would be depolarized and result in the propagation of action potentials in the afferent fibre. The met-enkephalin-like substance may be released tonically and either hyperpolarize the nerve terminal membrane or prevent transmitter release.

This model does not address the question of the function of the other peptide substances such as substance P and calcitonin-gene related peptide reported in mammalian Merkel cells (Hartschuh and Weihe, 1988). The significance of this complex array of substances requires further investigation.

CONCLUSIONS

The morphology of Merkel cell-neurite complexes is suggestive of a neurosecretory function. It has been shown that Merkel cells and their dense cored vesicles are required for the normal characteristic slowly adapting response of SA1 mechanoreceptors. Immunohistochemical studies indicated that there was a met-enkephalin-like substance present in rodent Merkel cells and that this was associated with the dense cored vesicles.

The work presented in this thesis shows that the

calcium channel blockers magnesium, cadmium, cobalt and verapamil hydrochloride inhibit the response of touch domes to mechanical stimulation. These results support the idea that release of a chemical transmitter is involved in the transduction process in SA1 mechanoreceptors.

Met-enkephalin also inhibits the response to mechanical stimulation and this effect is antagonized by naloxone. This indicates the presence of functional opioid receptors and suggests that the met-enkephalin-like substance acts as a modulator rather than a transmitter substance. The transmitter substance in mammalian Merkel cells remains to be identified but it is possibly glutamate or a related acidic amino acid.

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APPENDIX

APPENDIX

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CHAPTER 5

Calcium channel blockers and Merkel cells

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Introduction

It has been suggested for some time that slowly adapting type 1 (SA1) mechanoreceptors respond to mechanical stimulation by the release of a transmitter substance, possibly stored within Merkel cell dense cored vesicles. This concept was first suggested after the observation that there was a large number of dense cored vesicles between the nucleus and the subjacent nerve terminal (Iggo and Muir, 1969). The Merkel cell – neurite complex contains other ultrastructural characteristics which imply a neurosecretory function. Dense cored vesicles have been seen concentrated around areas of the Merkel cell membrane which are closely apposed to the subjacent terminal, where they have also been seen to fuse (Chen et al., 1973). Met-enkephalin-like immunoreactivity is associated with the dense cored vesicles of rodent Merkel cells (Hartschuh et al., 1979) and vasoactive intestinal polypeptide-like immunoreactivity is associated with dense cored vesicles of other mammalian Merkel cells (Hartschuh et al., 1983). Since these peptides are putative transmitters in other areas of the nervous system they may have a similar function in SA1 mechanoreceptors. More recent evidence indicates that the met-enkephalin-like substance is not the transmitter substance in rats but appears to have a modulatory function (Pacitti and Iggo, unpublished).

Analysis of the discharge pattern of SA1 mechanoreceptors shows that it is best described by an irregular oscillator model and the most likely cause of this is the release of transmitter substance from the Merkel cell (Horch et al., 1974).

Under hypoxic conditions feline SA1 mechanoreceptors failed progressively to respond to mechanical stimulation and this was correlated with a reduction in the number of dense cored vesicles in the Merkel cell, an effect reversed by return to normoxic conditions (Findlater et al., 1987). This is further evidence that the presence of dense cored vesicles is a requirement for the normal function of touch domes.

Calcium has been implicated in the transduction process in SA1 mechanoreceptors (Iggo and Findlater, 1984) since its presence is necessary for the release of a wide variety of molecules including neurotransmitters (Rubin, 1970). We have tested this idea using three calcium channel blockers in three experimental models.

Methods

Three experimental procedures were used to test electrophysiologically the effect of cobalt chloride ($CoCl_2$), cadmium chloride ($CdCl_2$) and verapamil hydrochloride on the response of SA1 mechanoreceptors to mechanical stimulation: (1) isolated hind limb perfusion in the cat; (2) intradermal injection under individual feline SA1 mechanoreceptors; (3) isolated superfused rat skin nerve preparation.

In models 1 and 2 cats were initially anaesthe-

tised with a 4% mixture of halothane in O_2 and anaesthesia was maintained using chloralose in saline (70 mg/kg i.v. of a 10 mg/ml solution). Single unit recordings of the responses to standardised mechanical stimuli were made from afferent fibres in the saphenous nerve and stored on an FM tape recorder (Ampex). Afferent fibres innervating the touch domes were electrically stimulated at 1.2 times threshold by inserting fine electrodes into the skin on either side of the receptor.

Model 1

A detailed description of this model has been published (Iggo and Findlater, 1984). Constant displacement mechanical stimulation (250 μm, probe tip diameter 0.5 mm) of suitable identified touch domes consisted of either (a) 1.5 s stimulus duration, 1 s interstimulus interval or (b) 5 s stimulus duration, 25 s interstimulus interval for a total time of 10 min. $CoCl_2$ (2.0, 3.0 and 5.0 mM) and verapamil (30 μM) were tested under conditions *a* above and $CdCl_2$ (0.5, 1.0 and 2.5 mM) under conditions *b*. The concentrations of drugs used are the estimated final concentrations circulating in the hind limb. These responses were compared with saline controls.

Model 2

Touch domes were stimulated as described under *a* above. $CoCl_2$ (125, 250 and 500 pM) and verapamil (0.01 pM) were injected under individual touch domes and the responses obtained compared to saline controls. Afferent fibres innervating the touch domes were stimulated as described above.

Model 3

Albino-Wistar rats were anaesthetised with urethane (25% w/v, 0.7 ml/kg i.p.) and supplementary doses given as required. A midline incision was made in the dorsal lumbar region exposing the 4th and 5th dorsal cutaneous nerves. One nerve was transected at its point of entry into the paraspinal muscles then it and the area of skin it innervated (10 × 20 mm) were dissected from the rat. The preparation was fixed epidermis uppermost onto a flat stainless steel mesh disc (26 mm diameter) located over a well in the superfusion chamber, with the nerve on a black perspex plate attached to the platform. The preparation was superfused (12 ml/min) with a physiological salt solution containing (mM): NaCl, 116; KCl, 5.4; $MgCl_2$, 1.2; $CaCl_2$, 2.5; dextrose, 5.6; HEPES, 10. The pH was adjusted to 7.4 prior to use and the solution heated to 31°C by passing it through a coil of polythene tubing sitting in a thermostatically controlled water bath surrounding the chamber. The epineurium was slipped back from the nerve. The tip of a glass micropipette was broken back so its internal diameter was slightly larger than the nerve's. The nerve was sucked into the pipette by creating a negative pressure with a 1 ml syringe attached to a polythene suction tube. A small amount of silicone grease was sucked into the space between the nerve and the pipette, thus creating a good seal which facilitated signal recording by improving the signal to noise ratio.

Touch domes were stimulated as described under *b* above. Appropriate quantities of drugs were added to the superfusing solution to give final concentrations of 0.05, 0.1, 0.5 or 1.0 mM $CdCl_2$ or 20, 50 or 100 μM verapamil. These responses were compared to control values.

Histology

In models 1 and 2 the hind limb was perfused with a 4% paraformaldehyde/2.5% glutaraldehyde/0.1 M cacodylate buffer fixative (pH = 7.4). Tissue was removed for normal processing for electron microscopical examination.

Data and statistical analysis

All data were analysed on a Cromemco System 3 microcomputer. Results were normalised to eliminate inherent variation between individual mechanoreceptors. All results are expressed as

mean ± SEM. Student's *t*-test was used for statistical analysis of the data.

Results

In all experimental models the overall effect of the calcium channel blockers was to decrease the response to mechanical stimulation.

Model 1, isolated hind limb perfusion

When 0.15 M NaCl was injected the response of the touch domes to mechanical stimulation never failed, even after 40 min of stimulation. With $CoCl_2$ the changes in the response of the SA1 mechanoreceptors were consistent between experiments but the time course of events varied. In all experiments there was a transient increase in afferent fibre activity within 30 s of the start of the injection which was unrelated to mechanical stimulation. Eventually the touch domes failed to respond to mechanical stimulation and the time to failure was concentration dependent: 2.0 mM caused failure in approximately 9 min (n = 3) and 3.0 and 5.0 mM caused failure in 3.5 – 4 min (n = 2). Measurement of serum Co^{2+} concentration showed that the peak value occurred around the time of receptor failure (0.97, 1.4 and 2.05 mM respectively). The effects of the two smallest doses were reversed by restoring the general circulation to the limb. In these experiments, though the response to mechanical stimulation had failed, there was still background activity associated with the afferent fibre under investigation.

When verapamil (30 μM) was injected the response declined gradually to 25% of control value after 10 min and fluctuated around this value for the rest of the experiment (n = 1).

$CdCl_2$ was given in a 1 ml bolus dose after the fifth mechanical stimulation. A summary of results is given in Table 1. One minute after injection, 0.5 mM $CdCl_2$ significantly increased the response of the touch domes to mechanical stimulation. With this exception $CdCl_2$ caused a dose dependent decline in the response of the SA1 mechanoreceptors. When the response to mechanical stimulation failed the afferent fibre still responded to electrical stimulation.

Model 2, intradermal injection

When 100 μl of 0.15 M NaCl was injected intradermally under the touch domes the response to mechanical stimulation continued for as long as the touch domes were stimulated. Injection of 125 and 250 pM of $CoCl_2$ caused a transient increase in the response of touch domes to mechanical stimulation. The time to receptor failure depended on the concentration: 125 pM reduced the response to 25% of controls after 6.5 min and 250 and 500

TABLE 1

Percent response at 1, 2, 5 and 8 min after $CdCl_2$ (0.5, 1.0 and 2.5 mM)

Time after $CdCl_2$ (min)	% response			
	Control	0.5 mM	1.0 mM	2.5 mM
1	99.6 ± 7.7	130.0 ± 8.7*	97.6 ± 15.9	65.4 ± 2.1**
2	93.4 ± 8.9	115.2 ± 3.0	88.4 ± 8.3	13.4 ± 7.0***
5	103.1 ± 7.6	100.9 ± 15.7	82.4 ± 0.9*	0.0 ± 0.0***
8	97.9 ± 8.8	95.4 ± 0.6	78.8 ± 3.8	0.0 ± 0.0***

Values are given as mean ± SE of mean; n = 5 for control response and n = 2 for others.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (*t*-test).

pM caused receptor failure at 3 min and 25 s respectively. When the response to mechanical stimulation failed, electrical stimulation of the afferent fibre innervating the touch dome still produced an action potential.

Histology

There was a significant decrease in the number of granules present in the cytoplasm adjacent to the nerve terminal in Merkel cells exposed to $CoCl_2$ and verapamil (Table 2). The remaining vesicles were generally found close to the cell membrane. There was also a significant increase in the number of 'synapse-like' structures observed in Merkel cells exposed to $CoCl_2$.

Model 3, isolated skin nerve preparation

Five stimulations were given before the preparation was exposed to drugs to ensure that the touch domes were responding in the usual way. $CdCl_2$ caused a dose dependent attenuation in the response of the SA1 mechanoreceptors to mechanical stimulation (Fig. 1). The higher concentrations caused the decline in response most quickly. Fifteen minutes after application the responses were reduced to 59.7 ± 7.9, 50.1 ± 2.7, 31.7 ± 10.4 and 23.0 ± 5.6% by 0.05, 0.1, 0.5 and 1.0 mM $CdCl_2$, respectively ($P<0.01$ at 0.05 mM and $P < 0.001$ at 0.1, 0.5 and 1.0 mM). Verapamil also reduced the response to mechanical stimulation dose dependently: 69.6 ± 1.7, 45.0 ± 4.5 and 31.6 ± 10.7% by 20, 50 and 100 μM ($P<0.001$) 15 min after application (Fig. 2).

TABLE 2

The vesicle density and the number of 'synaptic-like' structures in Merkel cells after saline, cobalt chloride or verapamil hydrochloride

Drug (mM)	Granule density (number/μm)		Synapses (number/section)	
Saline control	12.1 ± 1.5	(13)	0.6 ± 0.25	(13)
$CoCl_2$ (2.0)	4.3 ± 0.8**	(17)	2.1 ± 0.40*	(17)
Verapamil (0.03)	6.8 ± 0.8*	(18)	–	

Values are given as mean ± SE of mean, with number of observations in parentheses.
* $P < 0.01$, ** $P < 0.001$ (t-test).

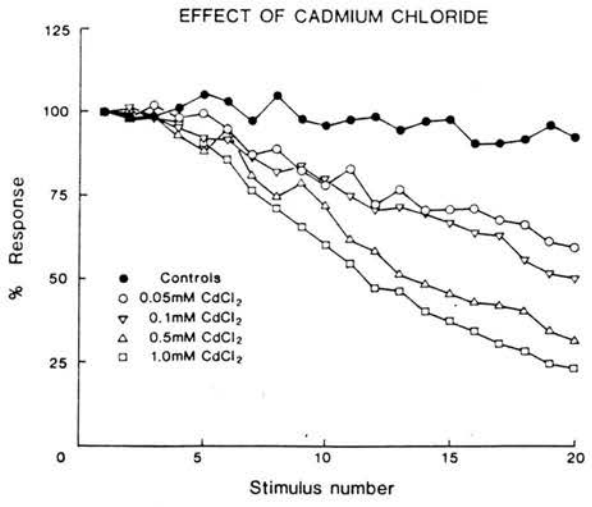

Fig. 1. Relationship between % response and stimulus number with increasing concentrations of cadmium chloride. Superfusing solution containing drug is given after stimulus 5. Stimuli are applied twice per minute for 5 s each. Response reduced after stimulus 8 ($P< 0.05$ at 0.05 mM, $P< 0.001$ at 0.1, 0.5 and 1.0 mM).

Discussion

It is now well established that several divalent cations including Co^{2+} and Cd^{2+} compete with Ca^{2+} for its channel binding site (Hurwitz, 1986). The dose dependent and reversible effect of Co^{2+}, with the exception of the highest concentration, on the evoked response of SA1 mechanoreceptors to mechanical stimulation is similar to that in the frog sciatic nerve–sartorius muscle preparation (Weakly, 1973; Kita and Van den Kloot, 1973). Increasing concentrations of Co^{2+} produced a progressive decrease in the end-plate potential (epp) which almost disappeared at bath concentrations of 1 mM Co^{2+}. This value is similar to the con-

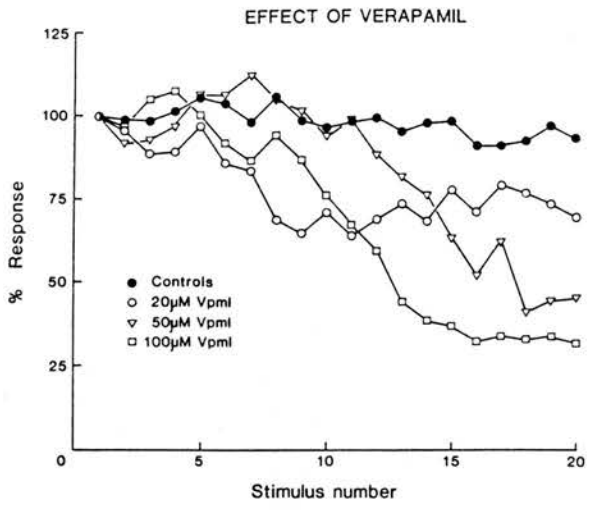

Fig. 2. Relationship between % response and stimulus number with increasing concentrations of verapamil (Vpml). Superfusing solution containing drug is given after stimulus 5. Stimuli are applied twice per minute for 5 s each. Response reduced after stimulus 8 ($P < 0.01$ at 20 and 100 μM) and stimulus 14 ($P < 0.01$ at 50 μM).

centrations measured in the blood serum when the touch domes failed to respond to mechanical stimulation. The effect of Co^{2+} was reversed by washing the preparation in a Co^{2+}-free solution and antagonised by elevating the external Ca^{2+} concentration (Weakly, 1973). Weakly (1973) suggested Co^{2+} inhibited neuromuscular transmission by competitive antagonism of Ca^{2+} leading to failure of transmitter release.

Cobalt (3 mM) also blocked the K^+ evoked release of substance P from cultured rat dorsal root ganglion neurones, [^{3}H]noradrenaline from cultured rat sympathetic neurones (Preney et al., 1986) and dopamine from isolated hypothalamic tuberoinfundibular neurones (Annunziato et al., 1986). The similarities between the effect of Co^{2+} in the Merkel cell – neurite complex and the neuromuscular junction (NMJ) and the demonstration that it can block evoked neurotransmitter release in vitro suggest that Co^{2+} may be blocking Ca^{2+} channels on the Merkel cell membrane, preventing release of a transmitter substance.

Cobalt produced an increase in activity in afferent fibres innervating the touch domes. Co^{2+} has been found to cause an increase in miniature end-plate potentials (mepps) produced by an increase in spontaneous transmitter release at the NMJ (Kita and Van den Kloot, 1973; Weakly, 1973). If Co^{2+} increased spontaneous transmitter release from each Merkel cell in a touch dome, then sufficient may be released to initiate action potentials in the afferent fibre.

In later experiments in model 1 and in all model 3 experiments, Co^{2+} was replaced by Cd^{2+} because it is a more selective Ca^{2+} blocker and to avoid the complications associated with Co^{2+}. The general effect of Cd^{2+} was very similar to Co^{2+}, i.e., a dose dependent decline in response, possibly due to prevention of transmitter release.

Acetylcholine or K^+ evoked release of catecholamines from perfused adrenal glands was inhibited by verapamil and methoxyverapamil (D600) at ID_{50}s of at least 10 μM (Arqueros and Daniels, 1978; Pinto and Trifaro, 1976). D600 (50 and 100 μM) inhibited the K^+ evoked release of dopamine from tuberoinfundibular neurones in vitro (Annunziato et al., 1986). Similar concentrations of verapamil reduced the response of touch domes to mechanical stimulation in our experiments, providing further evidence that transmitter release is involved.

At receptor failure there was a significant decrease in the number of dense cored vesicles present in Merkel cells after exposure to $CoCl_2$ or verapamil. If their only action was to block Ca^{2+} entry then an increase in the number of dense cored vesicles would be expected. In bullfrog dorsal root ganglion neurones it has been shown that fast axonal transport, but not protein synthesis, was inhibited by bathing the neurones in Co^{2+}-containing or Ca^{2+}-free solutions (Lavoie and Bennet, 1983). They suggested that Ca^{2+} was required for proteins to leave the Golgi region in transit for the fast axonal transport system. A similar process may operate in Merkel cells, thus preventing transit of proteins from the Golgi region.

Conclusions

These results suggest that Ca^{2+} is a necessary requirement for the normal function of SA1 mechanoreceptors, possibly via the Merkel cell. This is further evidence supporting the concept of transmitter release from Merkel cells.

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DENSE CORED VESICLES IN SAI MERKEL CELLS AND THEIR ROLE IN MECHANO-ELECTRIC TRANSDUCTION

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The SAI (slowly-adapting type I) mammalian mechanoreceptor is a well characterised and distinctive cutaneous sensory receptor. In hairy skin of the cat it is present as a dome-like epidermal structure innervated by a myelinated afferent fibre that ends in expanded disk-like flattened terminals each of which is associated with a Merkel cell (Iggo and Muir, 1969). Several studies have reported that the normal high sensitivity of the receptor to mechanical stimulation and the characteristic irregularities of the afferent discharge during sustained activity is dependent on the presence of innervated Merkel cells (Brown and Iggo, 1963; Burgess and Horch, 1973). The actual role of the Merkel cells in the normal function of the SAI is not yet, however, established and views range from the suggestion that they are the actual mechanoelectric transducers (Iggo and Muir, 1969; Horch et al., 1974) to the opposite extreme that they can be removed without affecting the functional properties of the sensory

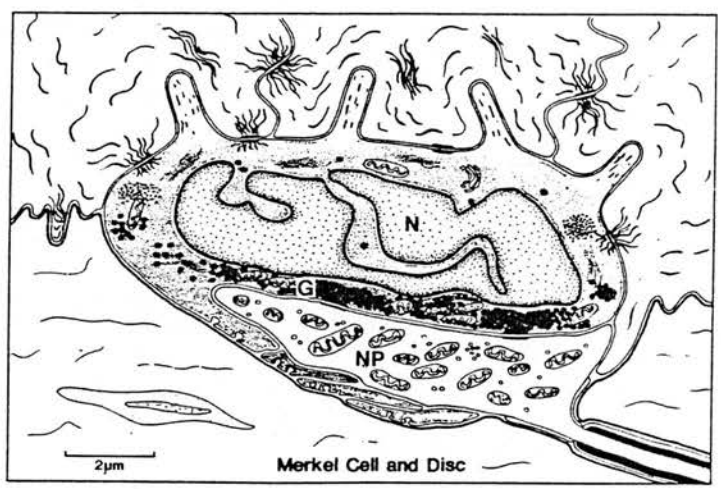

Fig. 1. Merkel cell-neurite complex, showing high density of granular vesicles (G) in Merkel cell cytoplasm between the cell's nucleus (N) and the expanded terminal of the nerve (NP).

receptor (Diamond, pers. comm.) or at least that they are not the transducers (Gottschaldt and Vahle-Hinz, 1982).

Any satisfactory account of the mechanism of the SAI mechanoreceptor must, however, provide an explanation for the functions of the various components that make up its normal structure (Fig. 1). These include, for example, the typical location of Merkel cells at the dermo-epidermal boundary, the additional layer(s) of epidermal cells that overly the Merkel cells, the various morphological features of the Merkel cells, the existence of special junctions between the Merkel cells and the subjacent expanded nerve terminal and so on. The present report deals with one particular aspect of the Merkel cells, namely the presence of a large number of encapsulated dense-cored vesicles in their cytoplasm. These vesicles are most abundant between the poly-lobulated nucleus of the Merkel cell and the subjacent nerve terminal (Fig. 1) and may be concentrated close to 'synapse-like' specialisations of the Merkel cell and nerve ending. If the vesicles were directly involved in the transfer of excitation between the Merkel cell and nerve-ending, that is, if the Merkel cell itself is the transducer then it might be expected that sustained mechanical stimulation of an SAI receptor would lead to depletion of granules. This has not, however, been reported and normally the granular vesicle content of the Merkel cells is apparantly unchanged when assessed by electron-microscopical examination of skin fixed immediately after prolonged mechanical stimulation. The SAI receptors, in fact, are capable of sustaining a more or less constant response to repeated intermittent mechanical stimulation (Findlater et al., 1987). Lack of information about the rate of formation and/or stability of the granular vesicles is at present an obstacle to reaching a conclusion, however, since if the rate of formation of vesicles was matched to their rate of release from the cell, or if only small numbers of vesicles were released during activity, the morphological assessment might be insufficiently sensitive to detect the changes.

In recent experiments on the metabolic requirements of the SAI receptors, in which their dependence on aerobic metabolism was examined (Findlater et al., 1987) it was found during severe hypoxia that the receptors became mechanically inexcitable at a time when the afferent fibre was still conducting normally. In these experiments, using anaesthetised cats and monkeys, SAI receptors were stimulated using quantitatively controlled mechanical indentations in conditions in which the atmosphere surrounding a severely hypoxic limb could be changed from 100% O_2 to 100% N_2. Exposure of the receptor to 100% N_2 progressively reduced the discharge in response to mechanical stimulation and finally extinguished it, and the process could be reversed by replacing the N_2 with O_2 (Fig. 2). The Merkel cells were examined electron-microscopically, in different preparations, at different stages of the cycle of O_2/N_2 replacement. In order to make valid comparisons, advantage was taken of the fact that SAI afferent fibres often innervate more than one cluster of Merkel cells. By choosing for study a unit that innervated at least two clusters or domes it was possible to compare mechanically stimulated and exhausted receptors with unstimulated receptors in otherwise identical conditions. The results obtained are tabulated in Table 1 and show clearly that there was a statistically significant reduction in the number of vesicles in the Merkel cells in exhausted receptors in hypoxic conditions when compared both with normal Merkel cells and with unstimulated cells in hypoxic conditions. This is the first time that it has been shown that the granular vesicle content of Merkel cells is labile, and although the results do not establish a causal relationship between the loss of vesicles and failure of mechanical excitability of the SAI receptors they certainly merit further investigation of the possibility that the Merkel cells are involved in transduction, whether directly in the series of

Table 1. Numerical density of granular vesicles in cytoplasm of Merkel cells from feline SAI receptors in different states of oxygenation

Experimental State	Vesicle no./μm^2 cytoplasm
Normal	14.6 ± 0.97 (n=17)
Hypoxic skin	
Not mechanically stimulated	8.3 ± 0.7 (n=16)
N_2 atmosphere, mech.stim.*	4.3 ± 0.48 (n=20)
O_2 atmosphere, mech.stim.*	7.9 ± 1.1 (n=7)

* = the receptors had ceased to respond to mechanical stimulation.

events leading to the discharge of afferent impulses or indirectly in some modulatory fashion.

Calcium channel blockers and SAI receptors

The results of the experiments using hypoxic conditions were further explored by examining the effects of calcium channel blockers on the mechanical excitability of SAI receptors and on the vesicle content of Merkel cells. The underlying reason for this approach was the fact that exocytosis of neurotransmitters requires the entry of calcium ions (Rubin, 1970; Baker, 1981) and therefore if indeed the release of dense-cored vesicles is an integral step in transduction then prevention of Ca^{2+} entry into the Merkel cell would reduce or abolish the response of the receptor to mechanical stimulation. In another situation where a secondary cell is

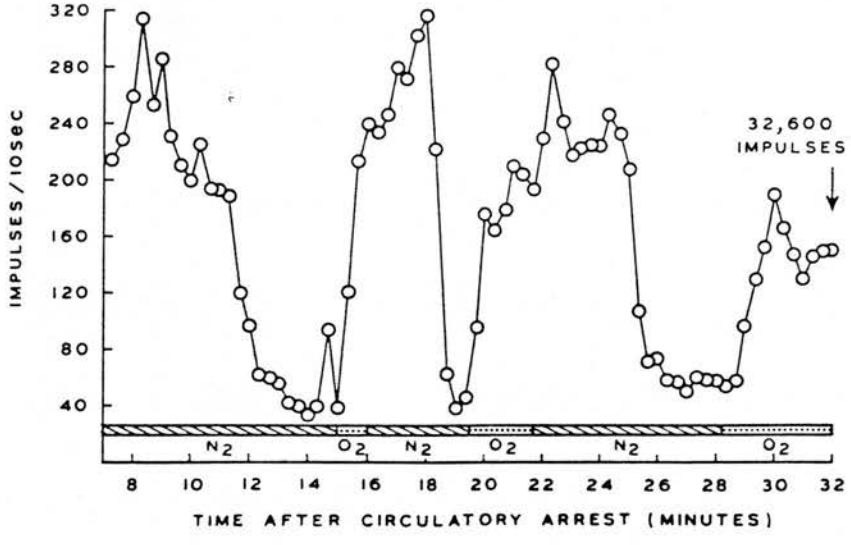

Fig. 2. Effect of hypoxia on mechanical excitability of an SAI mechanoreceptor in an anaesthetised monkey, during continuous intermittent mechanical stimulation. Graph begins 7 minutes after circulatory arrest. An N_2 atmosphere was formed around the limb at 7 minutes as indicated by the cross-hatched bar and periodically replaced by O_2.

Table 2. Numerical density and number of 'synaptic-like' structures in feline Merkel cells after exposure to normal saline, cobalt chloride or verapamil hydrochloride in the perfused hind limb circulation

Drug	No. of Vesicles/ μm^2	No. of "Synapses"/ Cell Section
Saline control	12.1 ± 1.5 (n=13)	0.6 ± 0.25 (n=13)
+$CoCl_2$ (2mM)	** 4.3 ± 0.8 (n=17)	*2.1 ± 0.40 (n=17)
++Verapamil (0.03mM)	* 6.8 ± 0.8 (n=18)	

* P<0.01 ** P<0.001 (t-test)
+ specimens examined after afferent response to mechanical stimulation was abolished
++specimens taken when afferent response to mechanical stimulation was 25% of control value

known to act as the transducer, the hair cell of the cochlea and lateral line system, Ohmori (1988) has established that calcium ions enter the apex of the hair cell of the chick cochlea when it is stimulated mechanically. These calcium ions then presumably lead to the release of a transmitter from the base of the hair cell and thereby cause excitation of the afferent nerve fibre. If a similar situation holds for Merkel cells, then block of calcium entry using a calcium channel blocker would be expected to prevent transfer of excitation from the Merkel cell to the afferent nerve terminal. Three kinds of calcium channel blocker were investigated - cobalt ions, cadmium ions and verapamil. The agents were either introduced into the isolated circulation of the cat hind limb (Findlater et al., 1987), injected intradermally under individual touch domes in anaesthetised cats or added to the perfusing solution in an isolated superfused rat skin preparation. At appropriate concentrations of the channel blockers the responses of SAI mechanoreceptors to mechanical stimulation in all three preparations were significantly reduced below control levels or even abolished. In contrast the electrical excitability of the afferent nerve fibres was unchanged. Ogawa and Yamashita (1988) report a similar reduction in mechanical excitability of frog slowly adapting cutaneous mechanoreceptors. The open question is whether the effect of the channel blockers was a direct action on the nerve terminal, or whether it was, at least in part, by an action on the Merkel cell. In Ogawo's experiments on frog skin it was possible to compare the effects of calcium channel blockers on both classes of slowly-adapting receptors, those corresponding to the SAI and SAII of mammals. Both kinds were affected, but to a greater degree and at lower concentrations of blocking agents for the SAI analogs. It was therefore of considerable interest to examine the Merkel cells in the mammals at a time when the responses to mechanical stimulation were reduced or even abolished. Contrary to expectations that the block of calcium entry would prevent the exocytosis of dense-cored vesicles there was actually a statistically significant reduction in the number of granules in Merkel cells removed at receptor failure in the presence of cobalt chloride and verapamil chloride (Table 2). At the same time there was an increase in the number of synaptic-like junctions between the Merkel cells and the nerve endings. Just as with hypoxia there was a correlation between the concentration of vesicles in the Merkel cells and the failure of mechanical excitability of the SAI receptors, in that at receptor failure there was a reduction in granule content of Merkel cells, but the results do not establish a causal relationship.

The exposure of the SAI receptors to cobalt chloride led initially to the spontaneous discharge of action potentials in the SAI afferent fibres, as well as an increased response to mechanical stimlulation, and it is thus possible that the cobalt ions were themselves responsble for the release of granules with consequent excitation of the afferent nerve terminal. The complexity of calcium actions in cells might indeed be such that the approach used in the present experiments is too indirect to give a clear-cut result. If, for example, calcium is required for the transport of vesicles from their site of formation in the Golgi apparatus, or even for the formation of the vesicles themselves (Lavoie and Bennet, 1983), then the reduction in vesicle numbers after exposure of the receptors to the channel blockers could be the consequence of both their reduced formation as well as the initial release of stored vesicles on exposure to cobalt chloride. In this case the reduced mechanical excitability of the receptors in the presence of channel blockers such as cobalt may result not only from lack of calcium entry limiting exocytosis but also from a diminished availability of dense-cored vesicles.

The results of the present investigation provide new evidence for an association between the presence/availability of dense cored vesicles in Merkel cells and the mechanical sensitivity of SAI cutaneuous mechanoreceptors in mammalian skin but do not provide conclusive evidence for the direct involvement of the Merkel cells in transduction or for the specific function of the dense cored vesicles.

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